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ORIGINAL PAPER

Transferrin and Lactoferrin – Human Iron Sources for Enterococci

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Abstract

To overcome limitations in iron acquisition, enterococci have evolved a number of mechanisms to scavenge iron from the host iron-binding proteins – transferrin (TR) and lactoferrin (LF). The aim of this study was to demonstrate the mechanisms by which enterococci utilize human TR and LF bound iron. The study included two strains of *Enterococcus faecalis* grown in iron-deficient and iron-excess media respectively. The binding activity of both proteins was monitored using proteins labelled with ^{125}I. The uptake of iron by enterococci was determined using ^{59}Fe labelled proteins. Reduction of iron bound to TR and LF was assayed with ferrozine. The proteolytic cleavage of TR and LF was visualized by SDS-polyacrylamide gel electrophoresis. The siderophore activity was measured with chrome azurol S. The study revealed that enterococci use several ways to acquire iron from TR and LF, such as iron chelating siderophores, iron reduction – facilitated iron release, protein degradation – promoted iron release, and receptor mediated capture of the iron-host protein complexes. The broad spectrum of iron acquisition mechanisms used by enterococci may play a significant role in the colonization of the human body and the resulting pathogenicity.

Key words: *Enterococcus* spp., iron acquisition, lactoferrin, siderophores, transferrin

Introduction

Members of the genus *Enterococcus* are widely distributed throughout nature as components of human and animal intestinal microbiota, and are also found in vegetables, plant materials and foods (Fisher and Phillips, 2009). Enterococci are typical opportunistic pathogens, and can cause urinary tract, wound, and soft tissue infections. They are associated with bacteremia which can lead to endocarditis in previously damaged cardiac valves (Gilmore *et al.*, 2013; van Tyne *et al.*, 2013). During the past several decades, enterococci, and particularly *Enterococcus faecalis* and *Enterococcus faecium*, have been identified as an important cause of nosocomial infections (Yuen and Ausubel, 2014). The major reason why these organisms survive in the hospital environment may be traced to their intrinsic natural resistance to several commonly used antibiotics. In the hospital setting, they can easily acquire other genes conferring resistance to many other classes of antimicrobial compounds (Gilmore *et al.*, 2013).

Iron is an essential element for bacteria, but is not easily available in host organisms. The concentration of free iron in humans is 10^{-18} M, an amount insufficient for maintaining the production of haem and non-haem iron-containing proteins. *In vivo*, bacteria have to contend with the natural ability of the host to withhold free iron by binding it to iron-protein complexes such as transferrin (TR) and lactoferrin (LF). TR is the iron carrier in the blood, while LF carries iron in secretory fluids. Competition for iron between the host and bacteria is an important factor determining the course of bacterial infections (Weinberg, 2009). At least four different mechanisms by which bacteria are said to acquire iron from host proteins have been suggested: Fe^{3+} chelating siderophore activity; reduction of carrier Fe^{3+} to the lower affinity iron Fe^{2+}resulting in its release; interaction of the Fe^{3+} carrier with a receptor on the cell surface; and proteolytic degradation of the carrier resulting in the splitting of the iron binding site (Krewulak and Vogel, 2008; Sheldon *et al.*, 2016).

Enterococci as lactic acid bacteria (LAB) for a long time were regarded as not requiring iron (Marcelis *et al.*, 1978). This concept, however, is changing in the light of the latest findings (Sobiś-Glinkowska *et al.*, 2001). To survive and multiply in the host, enterococci must possess efficient iron-acquisition mechanisms. It is believed that these mechanisms can be an

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important virulence factor for the enterococci. Still, little is known about enterococcal mechanism of iron assimilation. The goal of the present study was to demonstrate that enterococci may utilize human TR and LF as iron sources, and to detect which mechanism they use to acquire the iron.

Experimental

Materials and Methods

Bacterial strains. Two strains of *E. faecalis*, BD 122 and BD 123, were isolated from the blood of patients with endocarditis after cardiosurgery admitted to the Clinical Hospital No 1 in Lodz, then identified with API 20 Strep (bioMerieaux, France) and stored at –70°C in glycerol (Difco, USA).

Bacteriological media and growth conditions. Strains were grown in complex liquid medium (Lisiecki *et al.*, 1999). Iron deficient-medium was obtained using polyaminocarboxyl resin Chelex 100 (200–400 mesh, BioRad, USA). Strains were initially iron starved in liquid iron-deficient medium for 18 h at 37°C, and continuously shaken. The obtained culture was used to inoculate (5% v/v) a new portion of iron-deficient or iron-excess medium. After 24 h at 37°C with constant shaking, the culture was centrifuged (9500 g, 15 min, 4°C). The supernatant was filtered through a membrane filter (0.22 μm, Millipore, USA).

Determination of the MIC of EDDHA. The minimal inhibitory concentration (MIC) of ironchelator – ethylenediamine-di-o-hydroxyphenylacetic acid (EDDHA, Sigma, USA) was determined using the agar dilution method on Mueller-Hinton 2 medium (bioMerieux, France) (EUCAST, 2017).

Determination of ability to utilize iron bound in transferrin and lactoferrin. A standardized bacterial suspension (0.1 ml, $A_{580nm} = 0.1$) was added into 20 ml of melted Mueller-Hinton 2 agar medium with EDDHA, at concentration corresponding to MIC for the tested strains. The mixture was poured into plastic Petri dishes (Ø14 cm). After storing the plates for 24 h at 4°C, filter paper discs (No 3, Whatman, England) were placed on the surface of the plates and saturated with 5 μl of the iron binding host proteins, either TR (1000 μg) or LF (1000 μg, both from Sigma, USA). The negative control used was a disc with 1000 μg of human apo-transferrin (ATR) and apo-lactoferrin (ALF) (both from Sigma, USA). The control for bacterial growth consisted of a disc containing 25 μg of $FeSO_4 \times 7H_2O$ (Sigma, USA). The experiment was also performed with TR and LF filter paper discs separated from the bacteria by a 25,000 Da cutoff dialysis membrane (Visking Dialysis Tubing, Serva, Germany). Growth around the discs demonstrated the ability of the tested strains to utilize iron bound to host protein, and was assessed after 24 h and 48 h of incubation at 37°C.

Bacterial growth in human serum. Serum was obtained from the blood of healthy volunteers and inactivated for 30 min at 56°C. Approximately 10^6 viable iron-starved cells of each strain were added to 0.5 ml of, respectively, unsupplemented serum and serum containing 0.2 mM of ferric nitrilotriacetate (Fluka, Switzerland) to be later saturated with iron serum TR (Brock and Ng, 1983). After 18 h of incubation at 37°C, viable counts were calculated for each serum sample.

Transferrin and lactoferrin binding assay. Iron saturated TR and LF were labelled using the chloramine T method with $Na^{125}I$ (specific activity of 17.4 mCi/mg, NEN Life Science Products, USA) and Iodobeads (Pierce Chemicals, USA) (Markwell, 1982). Bacterial cells (10^9 CFU) were added to 1 ml of the liquid medium (without $MgCl_2$, $CaCl_2$, and glucose, but supplemented with 1 mg/ml of human albumin) and 10 μl of protein labelled with ^{125}I (3 μg protein, radioactivity approximately 10^3 cpm). After 2 h of incubation at 37°C, the suspension was centrifuged at 4500 g for 15 min at 4°C, and the bacterial pellet was washed three times with cold phosphate-buffered saline (PBS). The radioactivity associated with the bacteria was quantified using a gamma counter (Wallac, Sweden). Samples were tested in triplicate along with the control test tubes containing all ingredients except bacteria. The results were presented in units of nanograms of iodine-labelled protein bound per 10^9 bacterial cells (ng protein/10^9 CFU).

Uptake of iron from transferrin and lactoferrin. Human ATR and ALF labelling was performed with $^{59}FeCl_3$ (specific activity of 15.49 mCi/mg, NEN Life Science Products, USA) (Lindsey *et al.*, 1995). Bacterial cells (10^9) were mixed with 1 ml of iron-deficient medium (without $MgCl_2$, $CaCl_2$, and glucose) and 10 μl of protein labelled with ^{59}Fe (15 ng of iron, radioactivity of approximately 10^4 cpm). After 2 h of incubation at 37°C, the suspension was centrifuged at 4500 g for 15 min at 4°C, and the bacterial pellet was washed three times with cold PBS. The radioactivity retained in the bacterial pellet was measured using a gamma counter. Samples were tested in triplicate, along with the control test tubes containing all ingredients except bacteria. The results were presented in units of nanograms of ^{59}Fe bound per 10^9 bacterial cells (ng iron/10^9 CFU).

Iron reductase assay. The reduction of Fe^{3+} was assayed using ferrozine [3-(2-pirydyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine] (Sigma, USA) (Deneer *et al.*, 1995). Whole bacterial cells were suspended in 8 ml of medium (without $MgCl_2$, $CaCl_2$, and glucose), and 1 ml of the suspension was removed to measure its optical density at 580 nm. In the final volume of 7 ml, the reaction mixture contained: 50 μM NADH,

3 μM FMN, and 2 mM ferrozine (all reagents by Sigma, USA). After adding 100 μl of either TR or LF (100 μg/ml final concentration), the polystyrene tubes were shaken and incubated at 37°C. At 10, 20, and 30 min, 1 ml of reaction mixture was withdrawn, centrifuged (9500 g, 15 min, 4°C), and the absorbance at 562 nm was measured with a spectrophotometer (UV/VIS Cary 1, Varian, USA). The control tube contained all of the reagents except the enzyme source. The amount of Fe^{2+}-ferrozine complex was calculated as the difference between the absorbance values for the whole reaction mixture and the control mixture. Samples were tested in triplicate. The specific activity of ferric reductase was expressed as micromole Fe^{2+}-ferrozine complex formed per optical density unit (ODU) per minute (μmol Fe^{2+}-ferrozine/min/ODU). The results represent the mean values of three separate experiments.

Proteolytic activity of culture supernatants. Proteolytic activity was determined with gelatin and casein. Gelatin hydrolysis was evaluated on Todd-Hewitt agar (Difco) containing 3% gelatin. Casein utilization was analyzed on Trypticase Soya Agar (Difco) with 1.5% skim milk. Wells (Ø2 mm) were cut into the agar medium and 10 μl of the culture supernatants were pooled into these wells. Agar plates were incubated at 37°C for 24 h. The appearance of a turbid halo around the wells was considered positive for gelatin cleavage. The presence of a clear zone around the wells indicated caseinase activity (Kanemitsu *et al.*, 2001).

Proteolytic degradation of transferrin and lactoferrin. The culture supernatants and bacterial cells of the tested strains harvested from both the iron-deficient and iron-excess medium were tested. The culture supernatant (900 μl) was mixed with 100 μl of either TR or LF, to obtain a final concentration 1 mg/ml. After 2 h of incubation at 37°C, 20 μl was electrophoresed. Roughly 1×10^6 CFU/ml of whole cells was resuspended in 900 μl of iron-deficient medium (without $MgCl_2$, $CaCl_2$, and glucose). Either TR or LF (100 μl) was added to this suspension to a final concentration of 1 mg/ml. The mixture was incubated at 37°C for 2 h. After removing the bacteria with centrifugation (9500 g, 15 min, 4°C), 20 μl of the supernatant was subjected to electrophoresis. To visualize the destruction of LF or TR, SDS-polyacrylamide gel electrophoresis was performed with 12.5% resolving gels (Amersham Biosciences, Sweden). The gels were stained with Coomassie blue.

Determination of siderophores in the culture supernatants. Total siderophore activity in the culture supernatants was measured using chrome azurol S (CAS) (Schwyn and Neilands, 1987). Hydroxamate siderophores were assayed using a chemical-specific assay (Lisiecki *et al.*, 1999). The results were expressed as micrograms of desferrioxamine mesylate (Sigma, USA) per millilitre of culture supernatant.

Determination of iron concentration. Concentrations of iron in the medium were determined by spectrophotometric assaying, using iron test kits containing 1,10-phenanthroline (Merck, USA). Fe^{3+} was first reduced to Fe^{2+} by ascorbic acid, and the total amount of iron was measured as above.

Suspension density and viable count. The optical densities of suspensions and cultures were measured using a UV/VIS Cary 1 spectrophotometer at 580 nm, and standardized according to McFarland's scale. The viable count was estimated by using serial dilutions in PBS and standard plate methods on 4% Trypticase Soy Agar (Difco, USA).

Statistical analysis. Bacterial counts were compared with the non-parametric Mann-Whitney U test. Calculations were performed using the Statistica 7 (StatSoft®, Poland) software, and statistical significance was defined as $p \leq 0.05$.

Results

Multiplication of enterococci in human serum. Both the tested strains multiplied in Fe^{3+} non-enriched human serum (Table I). The serum enriched with Fe^{3+} to a concentration of 0.2 mM did not significantly ($p > 0.05$) stimulate the growth of the strains (Table I). The main iron binding-protein in human serum is TR.

Table I
Growth of enterococci in normal human serum

Strain	Inoculum (CFU/ml)	Unsupplemented serum iron (CFU/ml)	Supplemented serum iron (CFU/ml)
E. faecalis BD 122	1.9×10^6	3.8×10^6	3.0×10^6
E. faecalis BD 123	1.0×10^6	1.7×10^6	2.4×10^6

CFU, colony forming unit.

Ability of transferrin and lactoferrin to support the growth of enterococci. It was demonstrated, using the disc-diffusion technique, that human iron saturated TR and LF can support the growth of the tested enterococcal strains even under iron-deficient condition (Table II). Using apo-TR and apo-LF, the iron-free form of these proteins, instead of TR and LF did not promote growth of the tested enterococcal strains (Table II). The above results indicated that TR and LF can be used as iron sources for the tested enterococcal strains. Human TR and LF stimulated the acquisition of iron even when separated from the bacteria by a 25,000 Da cutoff dialysis membrane (data not shown). This experimental data suggests the involvement of siderophores – iron chelators – in the uptake of TR and LF bound iron. Only

Table II
Promotion the growth of enterococci by transferrin and lactoferrin

Iron sources	*E. faecalis* BD 122	*E. faecalis* BD 123
	Growth (mm)*	
None	0	0
Apo-transferrin	0	0
Transferrin	6	8
Apo-lactoferrin	0	0
Lactoferrin	7	9
Ferrous sulfate	10	11

* Details were described in the section of Materials and Methods. Response to iron-binding proteins was determined by the presence of a growth zone (mm) around a disc containing transferrin and lactoferrin. The negative control was disc containing apo-transferrin and apo-lactoferrin – iron-free form of these proteins. The control of bacterial growth consisted of a disc containing ferrous sulfate. The experiment was repeated twice and the results were consistent.

siderophores, as low molecular weight compounds with high affinity constants for iron, are able to overcome the barrier of a dialysis membrane, extract iron bound to TR and LF and provide them into bacterial cell.

Iron-chelators produced by enterococci. Under iron-deficient growth conditions, siderophore activity was detected in the culture supernatants of both strains, using a universal chemical assay with CAS. The growth medium contained the iron concentration of approximately 3.5×10^{-7} M. The siderophore activity of both strains was similar – 21.38 μg/ml and 19.71 μg/ml, respectively (Table III). The source of iron chelators, was a siderophore containing N-hydroxyamide groups belonging to the hydroxamate siderophore class (Table III). The enterococcal strains did not produce siderophores in the iron-excess medium (10^{-4} M).

Reduction of iron bound to transferrin and lactoferrin by enterococci. The iron bound to TR and LF was reduced by ferric reductases of whole cells in both strains (Table III). The culture supernatants did not exhibit this activity. Separation of the substrate from the cells by a dialysis membrane during incubation inhibited its reduction (data not shown). This finding indicates that the enterococcal ferric reductases are cell surface enzymes, and can be involved in enterococcal iron uptake.

Ability of enterococci to take up iron from transferrin and lactoferrin. To correlate the ability of TR and LF to support the growth of enterococcal strains with uptake of bound iron, the assimilation of iron from TR and LF was also tested. Whole cells of both strains actively acquired iron from the ^{59}Fe-labelled TR (Table III and IV). *E. faecalis* BD 122 acquired 57.5% and *E. faecalis* BD 123 acquired 50.7% of the initial isotope content, respectively. Iron from the ^{59}Fe-labelled LF was also absorbed by both strains, though to a considerably lesser extent: 3.1% of the initial isotope dose for strain BD 122 and 1.8% for strain BD 123 (Table IV).

Transferrin and lactoferrin binding activity of enterococci. Binding of TR and LF to the tested strains was quantified with iodine-labelled proteins. Human ^{125}I-LF was bound by the whole cells of both strains. *E. faecalis* BD 122 and BD 123 bound 2.5% and 2.4% of the initial isotope dose, respectively (Table III and V). The control value in these experiments was 33.00 ± 10.11 ng per 10^9 CFU, which corresponded to 1% of the initial isotope dose (Table V). Human ^{125}I-TR was not bound by the cells (Table III and V). The binding value for *E. faecalis* BD 122 was 30.00 ± 25.10 ng per 10^9 CFU, while for *E. faecalis* BD 123 it was 30.10 ± 13.20 ng per 10^9 CFU, amounting to 0.6% and 0.6% of the initial isotope doses, respectively. These values were identical with the control value (30.00 ± 15.10 ng per 10^9 CFU), corresponding to 0.6% of the initial isotope dose (Table V).

Table III
Suggested mechanisms of lactoferrin (LF) and transferrin (TR) bound iron acquisition by enterococci

Mechanism of iron acquisition		Strain	
		E. faecalis BD 122	*E. faecalis* BD 123
Hydroxamate siderophore (μg/ml)		21.38	19.73
Fe^{3+} reduction of carrier (μM Fe(II)-ferrozine/min/ODU*)	LF	0.10 ± 0.04	0.08 ± 0.05
	TR	0.01 ± 0.01	0.02 ± 0.01
^{125}I Carrier binding (ng protein per 109 CFU†)	LF	92.40 ± 19.9	79.20 ± 5.04
	TR	0	0
^{59}Fe iron-uptake (ng ^{59} Fe per 10^9 CFU)	LF	0.47 ± 0.23	0.28 ± 0.05
	TR	8.62 ± 1.17	7.6 ± 0.62
Proteolytic cleavage of iron bound to protein	LF	+	+
	TR	–	–

* ODU, optical density unit; † CFU, colony forming unit.

Table IV
Lactoferrin and transferrin binding by whole cells of the tested enterococci*

Protein	*E. faecalis* BD 122	*E. faecalis* BD 123	Control value
	ng iodine labelled protein per 10^9 CFU†		
Lactoferrin	82.50 ± 20.05	79.20 ± 15.08	33.00 ± 10.11
Transferrin	30.00 ± 25.08	30.07 ± 13.23	30.00 ± 15.12

* Cells were harvested from the iron-deficient medium. ^{125}I-labelled proteins were used. All experiments were completed using 1 ml of culture at 10^9 CFU/ml. Radioactivity is represented as ng of iodine labelled protein binding by the cells. The control mixture contained all of the reagents except the bacteria. Each column displays the mean values of three determinations from one experimental run.
† CFU, colony forming unit.

Table V
Iron uptake from lactoferrin and transferrin by whole cells of the tested enterococci*

Protein	*E. faecalis* BD 122	*E. faecalis* BD 123	Control value
	ng ^{59}Fe per 10^9 CFU†		
Lactoferrin	0.47 ± 0.23	0.28 ± 0.05	0.05 ± 0.03
Transferrin	8.62 ± 1.17	7.60 ± 0.62	0.29 ± 0.03

* Cells were harvested from the iron-deficient medium. All experiments were completed using 1 ml of culture at 10^9 CFU/ml. Radioactivity is represented as ng of ^{59}Fe transported into the cell. The control mixture contained all of the reagents except the bacteria. Each column displays the mean values of three determinations from one experimental run.
† CFU, colony forming unit

Proteolytic degradation of transferrin and lactoferrin by enterococci. Finally, the susceptibility of TR and LF to proteolytic degradation by the tested strains was investigated using SDS-polyacrylamide gel electrophoresis. The strains were proteolytically active towards the standard proteolysis substrates: casein and gelatin. Only the culture supernatants of both strains were active with LF. The products of degradation of LF into lower molecular weight fragments were detected after 2 h of incubation (Fig. 1 and 2). The excessive amount of Fe^{3+} (10^{-4} M) in the culture did not affect the proteolytic activity. Human TR was not proteolytically degraded by either strain in both the presence of the culture supernatants or whole cells (Fig. 1 and 2). The obtained data suggests that proteases active on TR are not produced by the tested enterococcal strains.

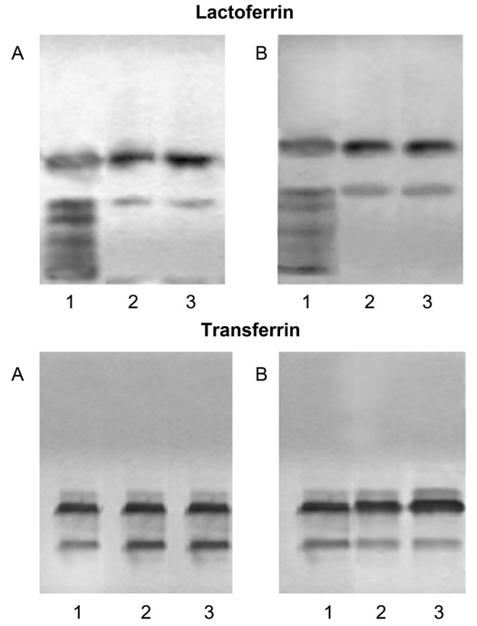

Fig. 1. SDS-polyacrylamide gel electrophoresis of the proteolytic degradation of iron-binding proteins – lactoferrin and transferrin by *E. faecalis* BD 122 grown in iron-deficient (A) or iron excess medium (B).

Lane 1, culture supernatant and iron-binding protein; lane 2, whole cell and iron-binding protein; lane 3, liquid growth medium and iron-binding protein. Approximately 20 µg of protein was separated on 12.5% polyacrylamide gel and stained with Coomassie blue.

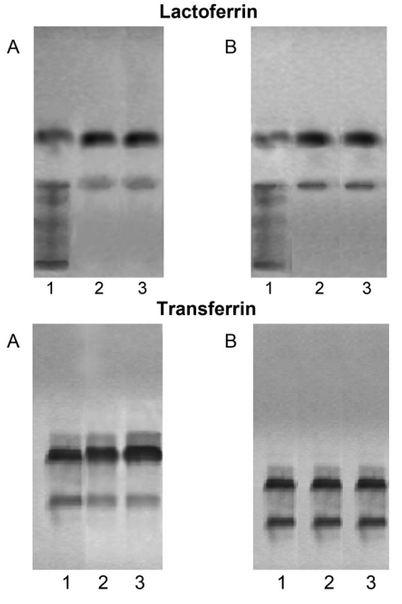

Fig. 2. SDS-polyacrylamide gel electrophoresis of the proteolytic degradation of iron-binding proteins – lactoferrin and transferrin by *E. faecalis* BD 123 grown in iron-deficient (A) or iron excess medium (B).

Lane 1, culture supernatant and iron-binding protein; lane 2, whole cell and iron-binding protein; lane 3, liquid growth medium and iron-binding protein. Approximately 20 µg of protein was separated on 12.5% polyacrylamide gel and stained with Coomassie blue.

Discussion

Numerous pathogenic and opportunistic bacteria utilize the human iron binding proteins – transferrin (TR) and lactoferrin (LF) as a source of iron (Mietzner and Morse, 1994; Morgenthau *et al.*, 2013; Parker Siburt

et al., 2012). Prior to the present work, there was no data in the available literature on *Enterococcus* spp. In this study, we evaluated the ability of two strains of *E. faecalis* to utilize human TR and LF as a sources of iron, and investigated the mechanism by which iron can be obtained from this protein. The tested enterococci strains acquired ^{59}Fe from human TR and LF. Iron saturated TR and LF were found to support growth of the tested strains. Bacteria growth or survival in human serum was, thus, associated with their ability to acquire iron from TR, the main source of iron in the blood (Mietzner and Morse, 1994). Lactoferrin has been identified in various secretory fluids, such as mammalian breast milk, saliva, tears, and mucosal secretions. Lactoferrin is also present in specific granules of neutrophils, and can also be find in stool, after being released from fecal leukocytes (García-Montoya *et al.*, 2012). Enterococci are inhabitants of the gastrointestinal tract of human microbiota (Fisher K. and C. Phillips, 2009). The bacteria can acquire iron not only from human TR and LF, but also from the iron carriers of other animals. Previous studies show that enterococci have the ability to acquire iron from ovotransferrin and bovine TR and LF (Sobiś-Glinkowska *et al.*, 2001).

Siderophores, extracellular iron chelators, are produced in response to an environmental iron deficiency by numerous, but not all, bacteria (Krewulak and Vogel, 2008; Kurth *et al.*, 2016.). The binding of Fe^{3+} by these compounds, with a stability constant (K) of 10^{22} to 10^{50}, is considered sufficient for iron uptake from body carriers such as TR, LF, and ferritin, but not from haem proteins (Harris *et al.*, 1979; Drechsel and Winkelman, 1997). The TR and LF stability constants at a pH of 7.4 (*i.e.* physiological conditions) equal $10^{25.6}$. Until now, enterococci have been regarded as not having the ability to produce siderophores. However, it has been stated that the bacteria produce iron chelators belonging to the hydroxamate class of siderophores (Lisiecki *et al.*, 1999). We demonstrated that production of the hydroxamate siderophores in the growth medium by both of the tested strains was induced by iron deficiency at concentration of 3.5×10^{-7} M. Even lower free iron concentration of the human body (10^{-18} M) may induce the production of hydroxamate siderophores, which may bind the iron of TR and LF and transport it into the cells.

Iron bound to TR and LF may be released by bacteria through reduction by assimilating the ferric reductases, which are not a part of the respiratory chain. The stability constant of Fe^{2+}-carrier complexes is then reduced to $K = 10^8$, and the complexes undergo dissociation, with the Fe^{2+} ion being released in a form easily assimilated by bacteria (Clarke *et al.*, 2001). Assimilation of iron by ferric reductases is widespread amongst bacteria living in aerobic environments with neutral pH (Fontecave *et al.*, 1994; Schröder *et al.*, 2003). The bacterial ferric reductases possess wide substrate specificities, and also reduce the Fe^{3+} of siderophores, TR, LF, or ferritin (Deneer *et al.*, 1995; Schröder *et al.*, 2003; Vartivarian and Cowart, 1999). Our results show the presence of ferric reductase activity toward TR and LF in the tested strains. The higher activity of reductases towards LF compared to TR is likely to be associated with the adaptation of enterococci for colonization of the digestive tract and vaginal vault mucosa, where LF is the most available iron source. Thus, the reduction of iron bound to TR and LF requires enterococcal cells to have direct access to these substrates. This access limits the involvement of reductases in the mobilization of iron sources from these iron carriers in the body. However, the majority of bacteria have only cell-related assimilating ferric reductases at their disposal (Schröder *et al.*, 2003).

Bacteria can acquire TR and LF bound iron to bind the protein on its surface *via* the specific receptor (Schröder *et al.*, 2003). Some reports have suggested that enterococci can bind to TR and LF (Zareba *et al.*, 1997; Styriak *et al.*, 2004). However, the investigated enterococci strains bound only iodine-labelled ^{125}I-LF, and acquired ^{59}Fe from both LF and TR. Therefore, it can be assumed that iron bound to LF might be assimilated into the cell after direct binding to the carrier through the receptor, whereas iron bound to TR, after reduction with ferric reductases, might be acquired in the form of Fe^{2+} through simple diffusion. Experimental conditions used in our experiments which utilized whole cells did not promote siderophore synthesis, which requires growth and multiplication of cells (Ratledge and Dover, 2000).

Bacterial extracellular proteases can similarly be involved in the iron uptake. These enzymes can cleave TR and LF into smaller fragments, and may result in the loosening of the Fe^{3+} bond to the protein, releasing the iron and allowing it to be more easily sequestered by siderophores. Iron (Fe^{3+}) can be also reduced to Fe^{2+} by ferric reductases, resulting in their release. The extracellular proteases in enterococci culture supernatants have cleaved only LF. The source of proteolytic activity of the enterococcal supernatants was probably gelatinase (Gel E). Both tested strains show proteolytic activity towards gelatine. Presence in enterococci of two additional proeteases, SprE and Deg P, was reported (Strzelecki *et al.*, 2011). It was also demonstrated that enterococci are proteolytically active towards haemoglobin – haem iron carrier (Sobiś-Glinkowska *et al.*, 2001).

To summarize, these experimental results are the first ever to indicate that human TR and LF are the source of iron for enterococci. The present study revealed that enterococci can use several strategies to acquire iron from TR and LF, such as iron chelating siderophores, iron reduction – facilitated iron release, receptor medi-

ated capture of the iron-host protein complexes and protein degradation – promoted iron release. It is possible that interactions exist between these mechanisms in enterococci. Enterococci used more mechanisms of iron aquisition from LF compared with those used to acquire TF bound iron. It probably results from the bacterial adaptation to niches settled in the organism. Bacteria of the genus *Enterococcus* are characterized by a natural and acquired resistance to many antimicrobial agents (Fisher and Phillips, 2009; Gilmore *et al*., 2013). For these reasons, treatment of enterococcal infections poses significant difficulties. It is crucial for us to intensify research into the mechanisms responsible for facilitating iron uptake by these pathogens. Inhibition of iron uptake in enterococci can be helpful in reducing and combating enterococcal infections. It is worth pointing out that it can be the next, new target for medication against infection caused by enterococci.

Acknowledgements

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ORIGINAL PAPER

The Prevalence of Exoenzyme S Gene in Multidrug-Sensitive and Multidrug-Resistant *Pseudomonas aeruginosa* Clinical Strains

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Abstract

Pseudomonas aeruginosa rods are one of the most commonly isolated microorganisms from clinical specimens, usually responsible for nosocomial infections. Antibiotic-resistant *P. aeruginosa* strains may present reduced expression of virulence factors. This fact may be caused by appropriate genome management to adapt to changing conditions of the hospital environment. Virulence factors genes may be replaced by those crucial to survive, like antimicrobial resistance genes. The aim of this study was to evaluate, using PCR, the occurrence of exoenzyme S-coding gene (*exo*S) in two distinct groups of *P. aeruginosa* strains: 83 multidrug-sensitive (MDS) and 65 multidrug-resistant (MDR) isolates. *Exo*S gene was noted in 72 (48.7%) of the examined strains: 44 (53.0%) MDS and 28 (43.1%) MDR. The observed differences were not statistically significant (p = 0.1505). *P. aeruginosa* strains virulence is rather determined by the expression regulation of the possessed genes than the difference in genes frequency amongst strains with different antimicrobial susceptibility patterns.

Key words: *Pseudomonas aeruginosa*, exoenzyme S, multidrug sensitive, multidrug resistant, virulence genes

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen with increasing role in nosocomial outbreaks. It produces multiple virulence factors that have been implicated in both-pathogenesis and bacterial quorum sensing. *P. aeruginosa* strains virulence factors may be linked to bacterial cell surface or released outside the cells. Exoenzyme S is a secreted protein toxin with ADP-ribosyltransferase and GTP-ase activity (Barbieri and Sun, 2004). This toxin is encoded by *exo*S gene located at 4303141–4304502 chromosome position of *P. aeruginosa* PAO1 strain (Stover *et al.*, 2000). Exoenzyme S is formed by 453 amino acids and released in type-III secretion system directly to the cytosol of the animal epithelial cells (Barbieri, 2000; Krueger and Barbieri, 1995; Rumbaugh *et al.*, 1999b). Its main biological activity is the protein synthesis inhibition in eukaryotic cells. Exoenzyme S inhibits also phagocytosis of bacterial cells by macrophages. In this manner, it can interfere in host immunological response (Barbieri, 2000; Frithz-Lindsten *et al.*, 1997). It is also able to induce apoptosis of lymphocyte-T cells by their DNA fragmentation (Bruno *et al.*, 2000).

There are no direct criteria for *P. aeruginosa* strains classification into multidrug-resistant (MDR) group in the relevant literature (Falagas *et al.*, 2006; Magiorakos *et al.*, 2012). Many different authors usually provide their own definitions established for their studies purposes. According to the definitions formed in 1994 by the American Cystic Fibrosis Foundation, MDR strains should express resistance to all drugs from at least two different therapeutic groups, *e.g.* beta-lactams, aminoglycosides and quinolones. Such criterion was used by Hill *et al.* (2005). A different definition had been used by Tam *et al.* (2005). According to them, strains should be classified as MDR while resistance to ceftazidime, imipenem, tobramycin and ciprofloxacin can be found. Similar criteria were used by Obritsch *et al.* (2004) in the analysis of antimicrobial sensitivity of *P. aeruginosa* strains derived from the patients of the intensive care units, isolated in the United States between 1993 and 2002. Hsu *et al.* (2005) used both criteria – established by American Cystic Fibrosis Foundation, and their own. These authors had chosen a few indicators of antimicrobial resistance: piperacillin/tazobactam, ceftazidime and cefepime, imipenem, gentamicin, amikacin/tobramycin and ciprofloxacin/levofloxacin. Strains

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classified as MDR were resistant to at least two out of the antimicrobial groups mentioned above.

The aim of this study was to determine the *exo*S gene frequency and its difference in multidrug-sensitive (MDS) and MDR *P. aeruginosa* strains groups.

Experimental

Materials and Methods

A total of 148 non-duplicated *P. aeruginosa* clinical strains (83 MDS and 65 MDR) collected in the Department of Microbiology of dr. A. Jurasz University Hospital No 1 in Bydgoszcz were included in the study. The strains identification was done on the basis of standard microbiological procedures.

For antimicrobial susceptibility tests, using disc-diffusion method on Mueller-Hinton Agar (Becton Dickinson), the following antimicrobials were used: ticarcillin, piperacillin, ticarcillin/clavulanate, piperacillin/tazobactam, ceftazidime, cefepime, aztreonam, imipenem, meropenem, gentamicin, tobramycin, amikacin, netilmicin, ciprofloxacin (Becton Dickinson). Results of the antimicrobial susceptibility tests were interpreted according to EUCAST Recommendations. *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 strains served as susceptibility testing quality controls. All the strains included into the study were, respectively, sensitive or resistant to all antimicrobials out of three groups: beta-lactams, aminoglycosides and fluoroquinolones and classified as MDS or MDR.

Bacterial genomic DNA was isolated with Genomic Mini kit (A&A Biotechnology), according to the manufacturer's protocol. In order to confirm the DNA isolation correctness and to avoid false-negative results all DNA samples were checked firstly: all of them were mixed separately with Loading Buffer DNA IV (AppliChem) and separated in 1% agarose gel (Bio-Rad) in 1 × TBE (Tris-Borate/EDTA, Bio-Rad) at 9 V/cm during one hour using MINI SUB™ DNA CELL (Bio-Rad) apparatus. After staining for 30 minutes in ethidium bromide solution and subsequent washing step for 20 minutes with deionized water, gels were visualized in UV light with Quantity One (Bio-Rad) program. DNA samples were then stored at 4°C until further use.

The prevalence of the exoenzyme S-encoding gene was determined by PCR. The amplification procedure was carried out according to Lanotte *et al.* (2004), in 0.2 ml test tubes (Eppendorf) in the final volume of 20 µl. *Taq* polymerase was used with the total activity of 1 U per sample in 1 × BD buffer, $MgCl_2$ at the final concentration of 1.5 mM (FirePol DNA Polymerase, Solis BioDyne) and dNTPs set at the final concentration of 200 µM (Solis BioDyne) were applied. Following primers were used: *exo*S F and *exo*S R, with sequences 5'→3': -CTTGAAGGGACTCGACAAGG- and -TTCA GGTCCGCGTAGTGAAT- (Integrated DNA Technologies), respectively, both at the final amount of 12.5 pmol per reaction. Isolated DNA samples were added subsequently. DNA isolated from *P. aeruginosa* PAO1 strain served as an amplification positive control. In the amplification procedure, thermal cycler GeneAmp® PCR System 2700 (Applied Biosystems) was applied the following conditions program: pre-amplification at 94°C for 3 minutes; amplification – 30 cycles, each consisting of: 94°C – 30 s, 53°C – 60 s, 72°C – 60 s; final elongation 72°C for 5 minutes. The obtained amplification products in the volume of 6 µl were mixed with Loading Buffer DNA IV (AppliChem) and separated in 1.5% agarose gel (Bio-Rad) in 1 × TBE (Bio-Rad), at 9 V/cm for 1.5 hour in MINI SUB™ DNA CELL (Bio-Rad) or SUB-CELL® GT (Bio-Rad). The 100–3000 bp DNA size marker (Solis BioDyne) was used. After staining for 30 minutes in ethidium bromide solution and subsequent washing for 20 minutes with deionized water, gels were visualized in UV light with Quantity One (Bio-Rad) system, photographed and stored. The gene identification was done on the basis of fragment size, as presented in Figure 1. Detection of a 504 bp product for the strain tested and the PAO1 control simultaneously was interpreted as a positive result.

Statistical analysis was performed using chi square test (χ^2) with $\alpha \leq 0.05$ to determine the significance of the difference in *exo*S gene frequencies between MDS and MDR strains groups.

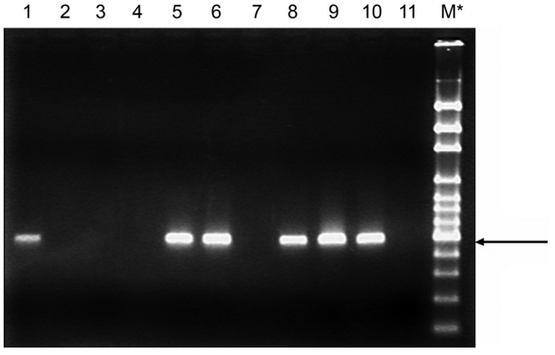

Fig. 1. An example of gel showing an electrophoretic separation of the *exo*S gene amplification product

* M-DNA size standard ranging from 100 to 3000 bp; 1–9 numbers of the examined strains; 10 – positive control; 11 – negative control; the arrow indicates the expected product size (504 bp).

Results and Discussion

In the present work *exo*S gene was found in 72 (48.7%) of the investigated *P. aeruginosa* strains. Positive results were obtained for 44 (53.0%) of MDS and

28 (43.1%) of MDR tested strains. An example is shown in Figure 1. The difference in *exo*S gene prevalence between both examined groups was not statistically significant ($p = 0.1505$).

Since couple of years, there have been some information on reduced virulence of MDR *P. aeruginosa* strains available in the scientific literature (Deptuła and Gospodarek, 2010; di Martino *et al.*, 2002; Ramisse *et al.*, 2000, Khosravi *et al.*, 2016). One of the explanations of this phenomenon is that bacteria cells somehow selectively silence some genes and activate other ones, currently more important from the survival point of view. However, in the available literature there are only a few reports on comparison of virulence factors genes expression in *P. aeruginosa* strains sensitive and resistant to several antimicrobials groups (Linares *et al.*, 2005; Fuse *et al.*, 2012). Linares and co-workers (2005) observed reduction of the type-III secretion compounds in *P. aeruginosa* after overexpression of particular multidrug efflux pumps. Interestingly, on the basis of the Fuse *et al.* studies (2012), synthesis of another *P. aeruginosa* important virulence factor and pigment-pyocyanin is also reduced in MDR strains. Moreover, its synthesis also decreases after metallo-beta-lactamases genes transduction into non-MDR *P. aeruginosa* strains. These facts could explain in what manner the more resistant strains cause infections with the lowest frequency.

A second explanation for the reduced virulence of MDR strains is the appropriate bacterial genome management that allows for survival in the antibiotic-supplemented environment. In the literature numerous researchers characterize *P. aeruginosa* genetic features in terms of different conditions, *e.g.* origin, clinical specimen, hospitalization time. However, still none information on virulence genes frequency in MDS and MDR groups of *P. aeruginosa* strains can be found in the relevant literature.

There is a wide diversity in the prevalence of *P. aeruginosa* genetic features. In the available positions the highest percentage of *P. aeruginosa* strains carrying *exo*S gene was found in the studies conducted by Tingpej *et al.* (2007) and Idris *et al.* (2012), reaching 100% and 93.2%, respectively. In contrary, Azimi *et al.* (2016) had recently confirmed the *exo*S gene presence only in 26.3% of the examined strains which is the lowest value ever mentioned.

Lanotte *et al.* (2004) studies revealed *exo*S gene presence in a range of 64.7% up to 93.8% of the examined strains. The values were related to the clinical specimen type which *P. aeruginosa* strains were isolated from. Amongst the clinical strains the lowest percentage was observed for the strains isolated from urine. One of the highest values was found in sputum- and lung-derived isolates. It would suggest the crucial role of exoenzyme S presence in pulmonary infections.

According to the results obtained by Khosravi *et al.* (2016), *exo*S gene was noted in almost 86% of the strains derived from patients' burns while in the work of Wolska and Szweda (2009) the gene was observed in 75.8% of the strains tested. *Exo*S gene in the study mentioned above was noted with the lowest frequency amongst all of the examined strains when compared to other virulence factors genes. The study of *exo*S gene prevalence including the highest number of *P. aeruginosa* strains was carried out by Pirnay *et al.* (2009). They had investigated 328 unrelated *P. aeruginosa* strains isolated during 125 years, in 69 places from 30 countries in 5 continents. The *exo*S gene was present in 72.6% of the strains. Those strains were isolated not only from hospitalized patients, but also from animals and environmental samples. Results similar to those obtained by Pirnay *et al.* (2009), but exclusively for clinical *P. aeruginosa* strains, using PCR and also Southern hybridization for the first one, were found by Feltman *et al.* (2001) and Garey *et al.* (2008). The gene was present in 72% and 70.5% of the examined strains, respectively. According to Fazeli and Momtaz (2014) the *exo*S gene is one of the most common (67.64%) virulence gene found amongst the tested strains. Similar level of the *exo*S gene presence (65.4%) among the examined *P. aeruginosa* strains was also noted by Yousefi-Avarvand *et al.* (2015). Another study done by Zhuo *et al.* (2010) detected *exo*S gene in 65.1% of the examined *P. aeruginosa* strains collected from patients from five hospitals. Almost 64% of *P. aeruginosa* isolates from ocular infections, examined by Choy *et al.* (2008) were also positive for *exo*S gene. Mitov and co-workers (2010) showed the presence of *exo*S gene amongst 62.4% of the examined *P. aeruginosa* strains but they did not confirm differences in the spread of the gene neither amongst MDR, nor non-MDR strains population. In the work published by Amirmozafari *et al.* (2016) the frequency of *exo*S gene reached 61% but none association was found between strains resistance and gene presence. The results demonstrated by Finlayson and Brown (2011) show that *exo*S genes presence is observed in approximately half of the examined strains while Winstanley *et al.* (2005) has confirmed *exo*S gene presence only in 38% of *P. aeruginosa* strains that had been examined. These latter values seems to be really close to the results obtained in this study where *exo*S gene was present in 43.1% up to 53.0% of the examined non-duplicated MDR and MDS strains, respectively.

Noteworthy, in the available literature, there is spare information on reduced *exo*S genes carriage amongst MDR *P. aeruginosa* strains to compare to our results. The only paper that includes similar research approach (Khosravi *et al.*, 2016) shows limited *exo*S gene presence in MDR comparing to overall strains population (77.1% *vs.* 85.8%) derived from burn patients.

Although, the aim of this study was only to estimate the *exo*S gene carriage, not the ability of exoenzyme S synthesis, it is also very interesting issue. Zhang and Wei (2009) study proved that the expression of virulence factors in *P. aeruginosa* strains is a very complex process. Exoenzyme S synthesis level may be for example raised by the glutathione concentration. Noteworthy, exoenzyme S is not always expressed, Tartor and El-Naenaeey (2016) detected its synthesis amongst 78.6% of *P. aeruginosa* strains. Similarly, results obtained by Tingpej *et al.* (2007) revealed that only 77% of the *exo*S gene-carrying strains synthesized this toxin in the respiratory tract infections studies. Meanwhile, during infection exoenzyme S production is necessary to invade *e.g.* epithelial cells, what is achieved by its injection directly into human cells (Heimer *et al.*, 2013; Hayashi *et al.*, 2015).

Joly *et al.* (2005), using RT-PCR, indicated increased *exo*S expression at the beginning of experimental *P. aeruginosa pneumonia* in rat model. The highest exoenzyme S transcript levels were present during the first two days of the infection. Its presence was linked with 29% mortality and, after two days, a drop in its synthesis was observed. Similar correlation in the animal model of *exo*S expression was indicated by Pierre *et al.* (2008). These authors claim that the expression of all genes connected with type-III secretion system decreases during chronic disease. In contrary to this conclusion, Hamood *et al.* (1996) and Rumbaugh *et al.* (1999a) claim that prolonged *P. aeruginosa* infection seems to increase exoenzyme S production. Moreover, it is synthesized significantly more often by wound- and urinary tract infections-derived *P. aeruginosa* strains (Hamood *et al.*, 1996).

Taken together, the results of this study indicate for the first time, although not statistically important, reduced exoenzyme S genes carriage in MDR *P. aeruginosa* strains when compared to MDS. All the facts mentioned above prove the advanced nature of *exo*S gene carriage, as well as expression, and indicate that further studies on that issue are necessary.

Conclusions

Exoenzyme S gene is not present in all *P. aeruginosa* strains, therefore not all of the strains have the ability to synthesize this virulence factor.

*Exo*S gene is noted more often amongst multidrug-sensitive *P. aeruginosa* strains, when compared to multidrug-resistant ones, but no statistically significant difference was observed.

The reduced virulence of multidrug-resistant *P. aeruginosa* strains is more likely caused by gene expression regulation, and not by the absence of virulence genes.

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ORIGINAL PAPER

Temperature, pH and Trimethoprim-Sulfamethoxazole Are Potent Inhibitors of Biofilm Formation by *Stenotrophomonas maltophilia* Clinical Isolates

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Abstract

Stenotrophomonas maltophilia, an opportunistic pathogen usually connected with healthcare-associated infections, is an environmental bacterium. Intrinsic resistance to multiple antibiotics, with different virulence determinants in the last decade classified this bacterium in the group of global multiple drug resistant (MDR) organism. *S. maltophilia* clinical isolates, were collected from tertiary care pediatric hospital in Belgrade, Serbia to investigate influence of different factors on biofilm formation, kinetics of biofilm formation for strong biofilm producers and effect of trimethoprim-sulfamethoxazole (TMP/SMX) on formed biofilm. Most of the isolates (89.8%) were able to form a biofilm. Analysis of biofilm formation in different growth conditions showed that changing of temeperature and pH had the stronggest effect on biofilm formation almost equally in group of cystic fibrosis (CF) and non-CF strains. TMP/SMX in concentration of 50 μg/ml reduced completely 24 h old biofilms while concentration of 25 μg/ml effects formed biofilms in a strain dependent manner. Among strains able to form strong biofilm CF isolates formed biofilm slower than non-CF isolates, while shaking conditions did not affect biofilm formation. Swimming motility was detected in both CF and non-CF isolates, however more motile strain formed stronger biofilms. This study suggests that temperature, pH and TMP/SMX had the strongest influence on biofilm formation in analyzed collection of *S. maltophilia*. A positive correlation between motility and strength of formed biofilm was demonstrated.

Key words: *Stenotrophomonas maltophilia,* biofilm, cystic fibrosis, opportunistic pathogen, trimethoprim-sulfamethoxazole

Introduction

Biofilm as a multicellular aggregation of bacterial cells is one mode of their organization, which provides several benefits for its members. The most important are survival in the unfriendly environment due to increased resistance to antibiotics and other antimicrobial agents (Balcázar *et al*., 2015). During biofilm formation, bacterial cells produce extracellular substances, which form a matrix as a single dynamic structure with different mutually interacting components (Payne and Boles, 2015). Although this extracellular matrix separates cells from their surroundings, bacterial cells could move freely from biofilm to planktonic form, which enables them to colonize surrounding biotic and abiotic surfaces. *Stenotrophomonas maltophilia* is a Gram-negative, multi-drug-resistant (MDR) opportunistic pathogen, which is usually connected with healthcare-associated infections (Brooke, 2012). Strains are usually isolated from water, soil, animals and plants and able to colonize different moist surfaces at homes and hospitals as well as surfaces of respiratory and urinary tracts. In addition, this pathogen possesses intrinsic and acquired resistance to various antibiotics (Sánchez, 2015). Recently it was classified in the emerging global opportunistic pathogens group (Brooke, 2014). The ability of *S. maltophilia* to form biofilm on different surfaces as well as its role in cystic fibrosis (CF) patients was analyzed previously (Pompilio *et al*., 2008; Pompilio *et al*., 2011). However, the real role of *S. maltophilia* in CF disease is still not clarified, although the latest data suggest that it comes to interspecies interactions between *S. maltophilia* and *Pseudomonas aeruginosa* as the main cause of CF infection (Sanders *et al*., 2010; Pompilio *et al*., 2015). In addition, the presence of *S. maltophilia* in the respiratory tract of CF patients forms specific conditions

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for *P. aeruginosa* chronic infection and/or increased virulence in mixed biofilm (Pompilio *et al.*, 2015).

In the last few years, *S. maltophilia* was frequently isolated at the Institute for Mother and Child Health Care "Dr Vukan Čupić", a University pediatric tertiary care hospital in Belgrade, Serbia mostly from the respiratory tracts of CF and non-cystic fibrosis (non-CF) patients. We investigated the ability of these strains to form biofilm under different growth conditions, kinetics of biofilm formation for strong biofilm producers and influence of trimethoprim-sulfamethoxazole (TMP/SMX) on formed biofilm of *S. maltophilia* isolates. The differences between CF and non-CF isolates are also assessed.

Experimental

Materials and Methods

Bacterial strains and media used. *S. maltophilia* clinical isolates were collected at The Institute for Mother and Child Health Care, a pediatric tertiary care hospital in Belgrade, Serbia. In total, 88 isolates were preliminarily identified using standard biochemical tests and automated Vitek 2 system (BioMérieux, Marcy l'Etoile, France), and confirmed with molecular identification by sequencing PCR products for 16S rRNA gene. Isolates were grown on Luria Bertani (LB) overnight at 37°C with aeration. All isolates were stored in LB with 15% glycerol at –80°C. *P. aeruginosa* PAO1 and *Escherichia coli* DH5α were also grown in LB media. For testing biofilm formation assay Trypticase soy broth (TSB) was used.

Biofilm formation and motility assay. Biofilm formation assay was performed as described previously (Stepanović *et al.*, 2007) with the slight modification described previously (Madi *et al.*, 2016). Biofilm samples were treated with 200 µl of a solution containing 96% ethanol and acetone in ration 4:1 for 15 min and the optical density (OD) was read at 595 nm with Microplate Reader (Tecan, Italy). The low cut-off (ODc) was calculated as the three standard deviations 3 × SD above the mean OD of control wells. Strains were classified according to the following criteria: no biofilm producer (OD ≤ ODc), weak biofilm producer (ODc < OD ≤ 2 × ODc), moderate biofilm producer (2 × ODc < OD ≤ 4 × ODc) and strong biofilm producer (4 × ODc < OD). Motility was analyzed as described previously (Madi *et al.*, 2016).

Influence of different factors on biofilm formation by *S. maltophilia*. Strains, which formed strong (7), moderate (36) and weak (5) biofilm were chosen for further analyses. Briefly, overnight cultures of *S. maltophilia*, prepared in TSB broth were washed twice, diluted with fresh TSB, and standardized to the density of 0.5 McFarland. Aliquots (200 µL) of standardized inoculums were added to the wells of sterile flat-bottom polystyrene 96 wells plates, incubation was performed in different conditions (pH (5.5 and 8.5), temperature (12, 30, 37, and 45°C), 10% saturation with CO_2 (Heracell 150, Thermo Fischer Scientific Inc., Walthman, MA, USA) biofilm formation was evaluated as described above in Biofilm formation assay paragraph. All experiments were performed in three independent repetitions.

Kinetics of biofilm formation. For seven strong biofilm producer strains kinetics of biofilm formation was determined. Aliquots (200 µl) of standardized inoculums were added to the wells of sterile flat-bottom polystyrene 96 wells plates and incubated at 37°C for 30 min, 1, 2, 4, 8, and 24 h. In addition, biofilm formation under dynamic conditions (shaking) was analyzed. Plates were rinsed, fixed and dyed as previously described, and the category of formed biofilm was calculated. Each assay was repeated three times.

The effect of trimethoprim-sulfamethoxazole on *S. maltophilia* formed biofilm. Biofilm formation by *S. maltophilia* was carried out as described above (Biofilm formation assay). After 24 h incubation at 37°C, the supernatant from each well was gently aspirated by micropipette; each well was then washed three times with 200 µl of 1X PBS and 200 µl of trimethoprim-sulfamethoxazole at two different concentration (25 and 50 µg/ml) were added to the wells. Controls were the formed biofilm by the strains without adding the antibiotic. Plates were incubated at 37°C for 6 h and biofilm was analyzed as described before. All experiments were performed in independent manner and repeated three times.

Statistical analysis. The statistical analysis and graph drawing were performed in R version 3.3.1. Heatmaps and cluster analysis was performed using R packages gtools, hclust and gplots. Statistical differences between the groups were assessed using t test.

Results and Discussion

The prevalence of *S. maltophilia* has increased in hospitals worldwide simultaneously with the appearance of a myriad of antibiotic resistant bacteria (Brooke, 2012, 2014). One of the usually present virulence factors in pathogenic bacteria and opportunistic pathogens is ability to form biofilm. Therefore, biofilm-associated infections substantially affect human health, increasing antibiotic resistance of bacteria and making it more challenging to combat such infections (Balcázar *et al.*, 2015). However, biofilm formation is influenced by different factors (Pompilio *et al.*, 2008; Di Bonaventura *et al.*, 2007).

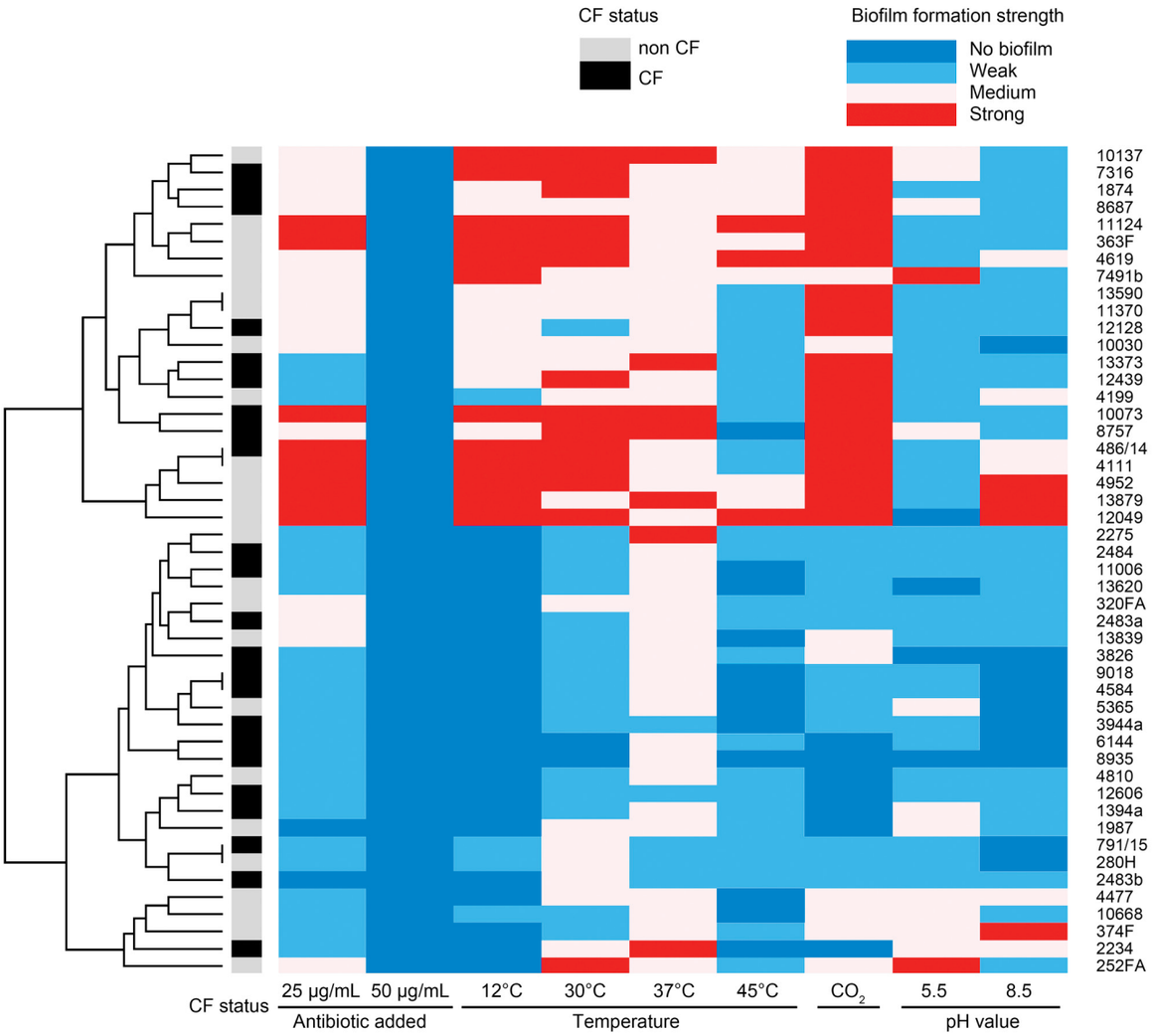

Fig. 1. Heatmap for biofilm formation under different growth conditions of *Stenotrophomonas maltophilia* clinical isolates. Conditions were grouped by antibiotic addition (first two), various temperature growth conditions, increased CO_2 and various environmental pH values.

Influence of different factors on biofilm formation by *S. maltophilia*. Previous results showed that among 88 *S. maltophilia* clinical isolates most of the strains were able to form a biofilm, both CF and non-CF isolates. Strong biofilm producers represented 7.95% and only nine strains (10.2%) did not form biofilm. For further analysis, we chose 48 strains (all strong and moderate biofilm producer as well as five weak biofilm producer). Results obtained for the influence of different factors on biofilm formation by selected *S. maltophilia* strains were present as a heatmap (Fig. 1). Additionally, hierarchical clustering was performed in order to acess the differences among the isolates abilities to form biofilm. Although isolate clusters slightly overlapped, all isolates are divided in four differentiated clusters in agreement with hierarchical clustering analysis. Clusters represent groups of isolates for which similar results in testing different factors on biofilm formation are obtained. Interestingly, both groups are present in the CF (black) and non-CF (gray) isolates suggesting that origin of strain did not influence the obtained results. Biofilm formation was the most affected with decrease or increase of temperature (12°C and 45°C) and changing pH to 8.5. The importance of the optimal temperature for biofilm formation was shown for the weak biofilm producer also (2483b, 791/15 and 280H), which formed a moderate biofilm at 30°C (Fig. 1). The temperature was showed to be the most relevant factor in biofilm formation by different strains not only in *S. maltophilia* but also in other bacterial species (Di Bonaventura *et al.*, 2007; Di Bonaventura *et al.*, 2008; The *et al.*, 2016). In addition, CF isolates were more sensitive to changes of temperature, pH and CO_2 concentration. Overproduction of thick and sticky mucus in patients with cystic

fibrosis forms a specific environment that certainly indirectly influences the characteristics of the bacteria that colonize it (Cantón and del Campo, 2010). This could be the reason for the slight difference in the characteristics of CF *vs.* non-CF isolates, because of adaptation to specific environmental conditions.

Effect of trimethoprim-sulfamethoxazole on *S. maltophilia* formed biofilm. *S. maltophilia* is intrinsically

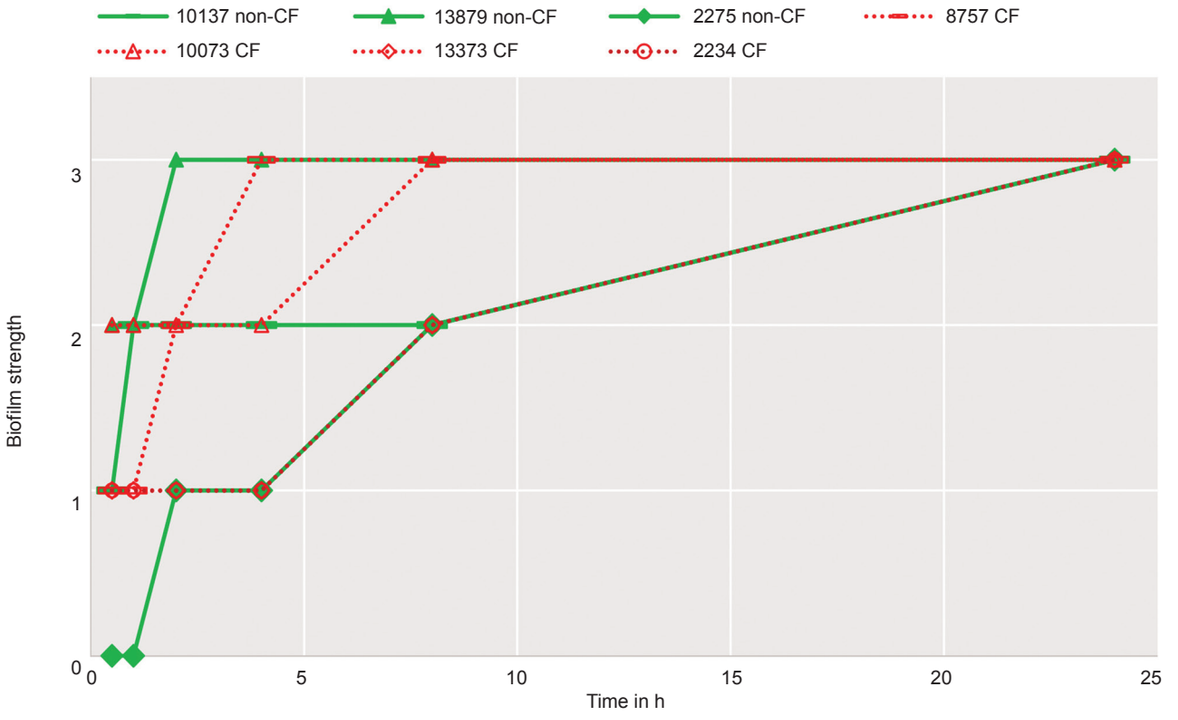

Fig. 2. Kinetics of biofilm formation by selected *Stenotrophomonas maltophilia* strains isolated from CF and non-CF patients. Biofilm strength was designated from 0 to 3 where 3 is strong, 2 is moderate, 1 is weak, and 0 is no biofilm.

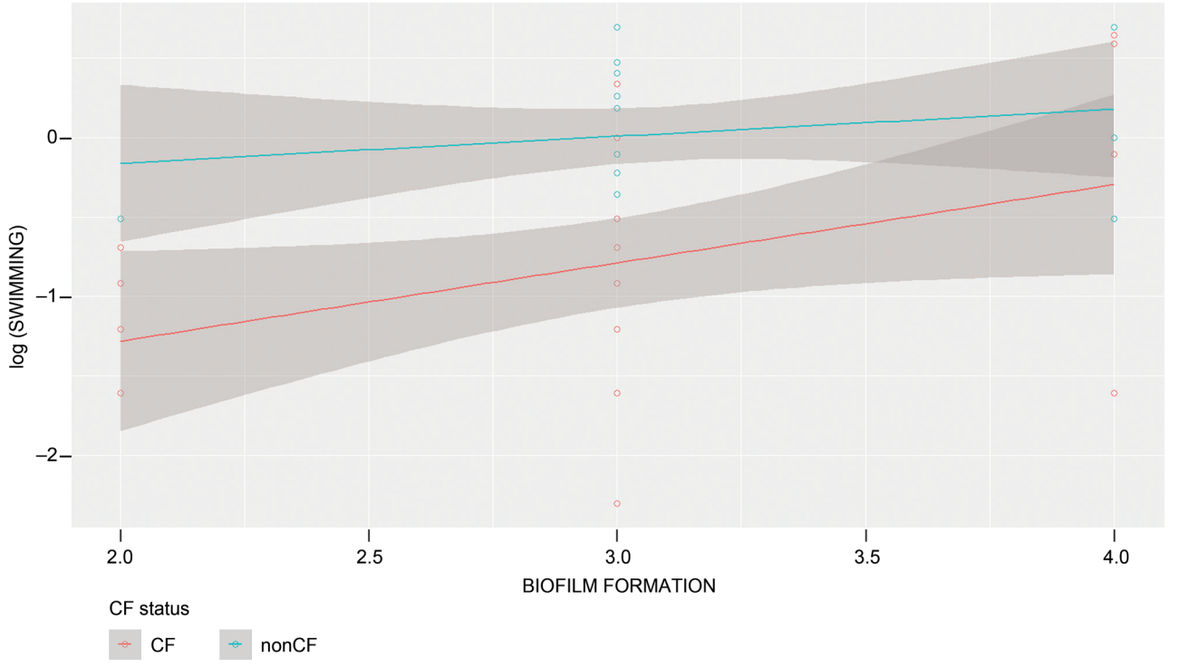

Fig. 3. Correlation between the strength of formed biofilm and motility of *S. maltophilia* strains isolated from CF and non-CF patients.

resistant to various antibiotics and so far, trimethoprim-sulfamethoxazole (TMP/MX) is recommended for the treatment of this bacterium (Abbott *et al.*, 2011). Although recently the number of reports of *S. maltophilia* resistance to TMP/SMX is increased (Hu *et al.*, 2016) we showed that in analyzed collection all isolates in their planctonic form were sensitive to it (Madi *et al.*, 2016). Once a biofilm has been formed, the bacterial cells become extremely robust against different antimicrobial agents. In this study, we further investigated the effects of TMP/SMX in two concentrations (25 μg/ml and 50 μg/ml) on biofilms formed by *S. maltophilia*. Both TMP/SMX concentrations were found to significantly contribute to the eradication of 24 h old biofilms (Fig. 1). However, cultivation of 24 h old biofilm with 50 μg/ml of TMP/SMX completely eradicated the formed biofilm in all tested strains. Lower applied concentration, 25 μg/ml of TMP/SMX affects the biofilm in a strain-dependent manner, from complete eradication to no, effect. Interestingly, strains more sensitive to other tested factors showed the higher sensitivity on 25 μg/ml of TMP/SMX.

Kinetics of biofilm formation. For selected strains, we determined the kinetics of biofilm formation (Fig. 2) which showed that non-CF isolates formed a biofilm faster than CF isolates. Although, they were all strong biofilm producers biofilm formation dynamic was significantly different between them correlating with the CF *vs.* non-CF phenotype. This might be, at least to some extent, attributed to the higher motility of non-CF isolates, which was shown previously (Madi *et al.*, 2016). In addition shaking conditions did not affect biofilm formation both by CF and non-CF isolates (data not shown).

Correlation between motility and strength of formed biofilm. All tested isolates showed swimming motility. We observed the same trend in changing motility and strength of formed biofilm (Fig. 3). Strains forming stronger biofilm showed higher motility with no statistically important differences in motility between CF and non-CF isolates ($p = 0.78$). Although, one study suggested that motility was important for biofilm formation in CF isolates (Pompilio *et al.*, 2015) in another lower motility in CF pathogens was described (Madi *et al.*, 2016). Thus, opposite results point to the complexity of the process of biofilm formation especially in the specific environment such as lung of CF patients.

Conclusion

The effect of analyzed factors on biofilm formation by *S. maltophilia* clinical isolates from CF and non-CF patents point out that there is no CF phenotype but we determined differences between these two groups of isolate. Complexity of this important virulence factor involves mutual influences of strains characteristics and environmental conditions. However, we could conclude that for the factors tested in this study temperature and pH had the strongest effect on strength of formed biofilm. A correlation between motility and biofilm formation was confirmed, a more motile strain formed stronger biofilm. In addition, TMP/SMX could easily eradicate the biofilm formed by the *S. matophila* clinical isolates tested in this study. Nevertheless, additional experiments are needed to completely evaluate mechanism of action of each factor on biofilm formation by this important opportunistic pathogen.

Acknowledgements

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Conflict of interests

The authors declare no conflict of interest.

Ethical standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the Ethics Committee of The Institute for Mother and Child Health Care (approval no. 8/6a) on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

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ORIGINAL PAPER

Prevalence, Influencing Factors, Antibiotic Resistance, Toxin and Molecular Characteristics of *Staphylococcus aureus* and MRSA Nasal Carriage among Diabetic Population in the United States, 2001–2004

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Abstract

Diabetic population were reported more likely to suffer carriage and infection with *Staphylococcus aureus* (*S. aureus*) and methicillin-resistant *Staphylococcus aureus* (MRSA) than non-diabetic population. We aim to elucidate the prevalence and characteristics of *S. aureus* and MRSA nasal carriage among diabetic population in the United States National Health and Nutrition Examination Survey, 2001–2004. Univariate analyses were conducted using Chi-square test, Fisher's exact probability test or student *t* test, as appropriate. Multivariate analysis using logistic regression was conducted to assess the association between influencing factors and *S. aureus* and MRSA nasal carriage. 1010 diabetic participants were included in the study. The prevalence of *S. aureus* and MRSA nasal carriage were 28.32% and 1.09%, respectively. After the logistic regression, ever had a painful sensation or tingling in hands or feet past three months (Odds Ratio [OR] = 0.359, 95% Confidence Interval [CI], 0.146–0.882) was significant among *S. aureus* nasal carriage and gender (OR = 3.410, 95% CI, 1.091–10.653) was significant among MRSA nasal carriage. The proportions of staphylococcal enterotoxin (SE) A, SEB, SEC, SED, Toxic-shock syndrome toxin-1, and Panton Valentine Leukocidin toxin among *S. aureus* strains were 18.75%, 3.13%, 12.50%, 15.63%, 28.13%, and 9.38%, respectively. 63.63% of MRSA strains were community-acquired, 27.27% were hospital-acquired, and 9.09% were non-typeable. Diabetic patients might be more likely to carry *S. aureus* and MRSA in the United States. Improving hand hygiene compliance, reducing antibiotic overuse, screening for carriers, and decolonization are recommended to reduce the spread of *S. aureus* and MRSA, especially in community.

Key words: *Staphylococcus aureus*, diabetics, MRSA, nasal carriage, NHANES

Introduction

Staphylococcus aureus (*S. aureus*) is a common pathogen and can cause different kinds of infection in both hospitals and communities (Knox *et al.*, 2015; Samanta *et al.*, 2015). Several parts of human body can carry *S. aureus* and the nasal cavity is the main part. It was reported that *S. aureus* nasal carriage can increase the possibility of infection (Wertheim *et al.*, 2005). Methicillin-resistant *Staphylococcus aureus* (MRSA) was first discovered in 1961 and then widely spread around the world (Chen *et al.*, 2012; Hernandez-Porto *et al.*, 2015). In the United States, the proportion of methicillin resistance in *S. aureus* strains approached almost 60% in 2003, with an average resistance rate of approximately 50% over the period from 1998 to 2002 (NNIS, 2004). In Europe, the proportion of methicillin resistance in *S. aureus* strains isolated from infected patients increased from 21% in 2002 to 23% in 2005 and then decreased to 19% in 2008. Between 2002 and 2005, the annual increase was 7.6%, followed by an average annual decrease of 4.8% (de Kraker *et al.*, 2013).

MRSA can increase the mortality rate, prolong hospitalization and aggravate the economic burden (Gastmeier *et al.*, 2012; Lodise and McKinnon, 2005). MRSA infection, hepatitis B and Acquired Immune Deficiency Syndrome has currently become three global infectious diseases.

Diabetes is a common chronic disease. With the development of social economy, the improvement of living standard and the aging of population, the number of diabetic population is increasing worldwide. The

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International Diabetes Federation Annual Report in 2014 showed that 387 million people were estimated to be living with diabetes, an alarming number that is set to rise to 592 million within the next twenty years. A further 316 million with impaired glucose tolerance are at high risk from the disease, with projections indicating that over one billion people will be living with or at high risk of diabetes in 2035 (IDF, 2014). Due to poor long-term blood glucose control or various complications, diabetic population is prone to suffer *S. aureus* or MRSA infection, which can cause death to a certain extent. Several studies reported that diabetes can increase *S. aureus* and MRSA carriage and infection (Chen and Pass, 2013; Daeschlein *et al.*, 2015). In other words, diabetic population was more likely to suffer carriage and even infection with MRSA than non-diabetic population.

There were a few studies focusing on *S. aureus* and MRSA nasal carriage among diabetic population and the conclusions were different. A Turkey study reported that the rates of *S. aureus* and MRSA nasal carriage among diabetic outpatient population were 41.9% (127/304) and 0.87% (30/304), respectively (Kutlu *et al.*, 2012). An Indian study reported that the *S. aureus* nasal carriage rate among hospitalized diabetic patients was 56.6% (34/60) (Ahluwalia *et al.*, 2000). A Chinese study reported that the rates of *S. aureus* and MRSA nasal carriage among hospitalized diabetic patients were 20.5% (41/200) and 0.50% (1/200), respectively (Junhua *et al.*, 2005). These studies, however, mainly focused on the prevalence and risk factors of *S. aureus* and MRSA nasal carriage among diabetic population, which were not comprehensive. Therefore, the objective of this current study was to elucidate the prevalence, influencing factors, antibiotic resistance, toxin, and molecular characteristics of *S. aureus* and MRSA nasal carriage among diabetic population who participated in the United States National Health and Nutrition Examination Survey (NHANES), 2001–2004.

Experimental

Materials and Methods

Study design and population. The continuous NHANES is a large, annual, cross-sectional survey that is designed to evaluate the nutrition and health status of the US population. The surveys were approved by the National Center for Health Statistics Research Ethics Review Board. Data releases are available in two-year increments. The *S. aureus* and MRSA nasal carriage data were only collected from this NHANES 2001–2004 dataset, so we used these data for analyzing. All participants provided written informed consent and the research ethics boards of the National Center for Health Statistics approved all protocols. More details regarding the informed consent of subjects, survey design, data collection, data procedures and laboratory assessment can be found elsewhere (CDC). Those who were tested the assessment of *S. aureus* and were self-reported diabetes were included in the current study.

Assessment of prevalence of *S. aureus* and MRSA. Assessment of *S. aureus* was conducted for the entire sample, thus, all participants aged one year or older were tested. Specimens collected from the nares were plated on mannitol salt agar, a selective medium for the isolation of *S. aureus*. After overnight cultures, specimens were used to perform Staphaurex and tube coagulase test. Staphaurex-positive and tube coagulase-positive isolates were identified as *S. aureus* and saved for further testing. *S. aureus* isolates were screened for methicillin resistance by the disk diffusion method of the National Clinical and Laboratory Standards Institute.

Diagnosed diabetes status. Diagnosed diabetes was identified in participants who answered "yes" to the self-reported question, "Have you ever been told by a doctor or health professional that you had diabetes?" and/or who used diabetes medication *(i.e.* oral hypoglycemic agents and/or insulin).

Potential influencing factors. Several variables were investigated as potential characteristics indicative of nasal carriage of *S. aureus* and MRSA among diabetic population. These factors included the self-reported diabetic characteristics variables, self-reported demographic variables, body measures variables, self-reported current health variables, self-reported medical conditions variables, self-reported alcohol use variables, self-reported drug use variables, self-reported physical activity variables, self-reported prescription medications variables, and self-reported exposure to cigarette smoke (operationalized as either being a smoker or living in a house with a current smoker). Given the limited population carriage of MRSA, we investigated a limited number of characteristics based on whether the sample size was large enough to make a reliable population estimate.

Assessment of antibiotic resistance of *S. aureus* and MRSA. Antibiotic resistance of *S. aureus* and MRSA was through antibiotic susceptibility testing by broth microdilution using Clinical and Laboratory Standards Institute reference methods. We calculated the resistance of 18 common antibiotics including tetracycline, clindamycin, erythromycin, penicillin, imipenem, vancomycin, cefazolin, oxacillin, gentamicin, ciprofloxacin, levofloxacin, rifampin, amoxicillin, chloramphenicol, doxycycline, daptomycin, quinupristin-dalfopristin, and linezolid. Moreover, we calculated multidrug resistance (MDR), which was defined as resistant to three or more antibiotics with different mechanisms of action

(note that these strains are already resistant to all beta-lactam antibiotics).

Assessment of toxin characteristics and molecular characteristics of *S. aureus* and MRSA. Strain typing by singleplex polymerase chain reaction (PCR) for detection of staphylococcal enterotoxins (SEs), toxic shock syndrome toxin-1 (TSST-1), Panton-Valentine Leukocidin (PVL) toxin, and staphylococcal cassette chromosome *mec* (SCC*mec*) type. SCC*mec* type is an important classification of MRSA that can be used to trace the source of the bacteria. According to some international reports, SCC*mec* I–III are designated as hospital-acquired (HA), SCC*mec* IV and V are designated as community-acquired (CA), and others are designated as non-typeable (NT) strains (Ito *et al.*, 2003; Okuma *et al.*, 2002). Although there have been studies (Freitas *et al.*, 2010) that reported possible misclassification errors for this typing, we still referenced it because there are no other accurate classifications.

Statistical analysis. Means and standard errors were calculated for continuous variables, and frequencies (percentages) were calculated for categorical variables. Univariate analyses were conducted using Chi-square test, Fisher's exact probability test or student t test, as appropriate. Multivariate analysis using logistic regression was conducted to assess the association between influencing factors and *S. aureus* and MRSA nasal carriage. We also conducted a logistic regression analysis of all variables with a P value of < 0.10 and then removed variables with a P value of ≥ 0.10. All statistical analyses were two-sided. All the statistical analyses were performed using Stata 13.1 (College Station, Texas, USA).

Results

Prevalence of *S. aureus* and MRSA nasal carriage. According to the inclusion criteria of study population there were 1010 diabetic population included in the current study. The prevalence of *S. aureus*, MRSA, and methicillin-sensitive *Staphylococcus aureus* (MSSA) nasal carriage were 28.32% (286/1010), 1.09% (11/1010), and 27.23% (275/1010) respectively.

Influencing factors of *S. aureus* and MRSA nasal carriage. We found that military status ($P = 0.027$), marital status ($P = 0.001$), ever still had a liver condition ($P = 0.033$), ever had cancer or malignancy ($P = 0.038$), ever blood relatives had diabetes ($P = 0.030$), ever taken antibiotics past month ($P = 0.017$), ever taken diabetic pills to lower blood sugar ($P = 0.040$), ever had numbness ($P = 0.071$), ever had a painful sensation or tingling in hands or feet past three months ($P = 0.026$), number of days used street drugs over past year ($P < 0.001$), and ever had five or more drinks every day ($P = 0.043$) were associated with *S. aureus* nasal carriage among diabetic population in this current study.

Gender ($P = 0.068$), number of alcoholic drinks per day over past 12 months ($P = 0.048$), number of days had five or more drinks past 12 months ($P = 0.036$), ever had vigorous activity past 30 days ($P = 0.070$), ever had moderate activity past 30 days ($P = 0.081$), frequency of playing or exercising hard every week ($P = 0.050$), and number of hours using TV, video or computer every day ($P = 0.002$) were associated with MRSA nasal carriage among diabetic population in this current study. More details can be found in Table I and Supplementary materials.

To account for potential confounding among the influencing factors, we further analyzed the relationship between the potential predictors with a logistic regression model. This model showed that when controlling for the effect of the other influencing factors, the relationships found in the univariate analyses changed. Ever had a painful sensation or tingling in hands or feet past three months was still associated with *S. aureus* nasal carriage among diabetic population in this logistic regression model. Having a painful sensation or tingling in hands or feet past three months was a protective factor. Having a painful sensation or tingling in hands or feet past three months among diabetic population were 0.359 times (95% Confidence Interval [CI], 0.146–0.882) less likely than those not having a painful sensation or tingling in hands or feet past three months to carry *S. aureus*. Gender was still associated with MRSA nasal carriage among diabetic population in this logistic regression model. Female was a risk factor. Female diabetic population was 3.410 times (95% CI, 1.091–10.653) more likely than male diabetic population to carry MRSA. More details can be found in Table II and Table III.

Antibiotic resistance of *S. aureus* nasal carriage. Among the proportions of antibiotic resistance in MRSA strains, penicillin (100.00%), imipenem (100.00%), cefazolin (100.00%), oxacillin (100.00%), and amoxicillin (100.00%) were the highest, followed by erythromycin (72.73%), tetracycline (27.27%), levofloxacin (27.27%), clindamycin (18.18%), chloramphenicol (11.11%), vancomycin (0.00%), gentamicin (0.00%), ciprofloxacin (0.00%), rifampin (0.00%), doxycycline (0.00%), daptomycin (0.00%), quinupristin-dalfopristin (0.00%), and linezolid (0.00%). Specially, there was no MRSA strain resistant to vancomycin, gentamicin, ciprofloxacin, rifampin, doxycycline, daptomycin, quinupristin-dalfopristin, and linezolid. Among the proportions of antibiotic resistance in MSSA strains, erythromycin (90.48%) and penicillin (90.48%) were the highest, followed by tetracycline (0.00%), vancomycin (0.00%), clindamycin (0.00%), imipenem (0.00%), cefazolin (0.00%), oxacillin (0.00%), gentamicin (0.00%), ciprofloxacin

Table I
Significant influencing factors of *Staphylococcus aureus* and MRSA nasal carriage

Influencing factors	Number of population	*Staphylococcus aureus*			MRSA		
		Number (%)	χ^2	P	Number (%)	χ^2	P
Gender			–	–		3.340	0.068
Male	553	155 (28.03)			3 (1.94)		
Female	534	131 (24.53)			8 (6.11)		
Military status			4.868	0.027		–	–
Yes	77	16 (20.78)			1 (6.25)		
No	507	169 (33.33)			6 (3.55)		
Marital status			20.062	0.001		–	–
Married	277	105 (37.91)			3 (2.86)		
Widowed	55	12 (21.82)			0 (0.00)		
Divorced	46	11 (23.91)			2 (18.18)		
Separated	13	2 (15.38)			0 (0.00)		
Never married	252	58 (23.02)			2 (3.45)		
Living with partner	34	14 (41.18)			0 (0.00)		
Do you still have a liver condition			4.530	0.033		–	–
Yes	27	3 (11.11)			0 (0.00)		
No	25	9 (36.00)			0 (0.00)		
Ever told you had cancer or malignancy			4.310	0.038		–	–
Yes	147	32 (21.77)			1 (3.12)		
No	828	250 (30.19)			10 (4.00)		
Blood relatives have diabetes			4.723	0.030		–	–
Yes	717	222 (30.96)			9 (4.05)		
No	230	54 (23.48)			2 (3.70)		
Which biological [blood] family member have diabetes?			–	–		–	0.093
Mother	114	31 (27.19)			1 (3.23)		
Father	63	19 (30.16)			0 (0.00)		
Mother's mother	31	6 (19.35)			2 (33.33)		
Mother's father	17	6 (35.29)			0 (0.00)		
Father's mother	20	8 (40.00)			1 (12.50)		
Father's father	9	3 (33.33)			0 (0.00)		
Brother	90	29 (32.22)			2 (6.90)		
Sister	204	68 (33.33)			2 (2.94)		
Other	167	51 (30.54)			1 (1,96)		
Taken antibiotics past month			5.695	0.017		–	–
Yes	54	8 (28.89)			1 (12.50)		
No	576	174 (30.21)			5 (2.87)		
Take diabetic pills to lower blood sugar			4.229	0.040		–	–
Yes	684	208 (30.41)			6 (2.88)		
No	323	78 (24.15)			5 (6.41)		
Has the numbness been in hands, feet, or both			5.287	0.071		–	–
Hands	92	20 (21.74)			0 (0.00)		
Feet	116	41 (35.34)			1 (2.44)		
Both	144	37 (25.69)			2 (5.41)		
Had a painful sensation or tingling in hands or feet past three months			4.954	0.026		–	–
Yes	353	87 (24.65)			5 (5.75)		
No	562	177 (31.49)			5 (2.82)		

Table I continued

Influencing factors	Number of population	*Staphylococcus aureus*			MRSA		
		Number (%)	χ^2	P	Number (%)	χ^2	P
Number of days used street drugs over past year			43.144	< 0.001		–	–
< 30	67	20 (29.85)			0 (0.00)		
30 –	0	0 (0.00)			0 (0.00)		
90 –	0	0 (0.00)			0 (0.00)		
180 –	0	0 (0.00)			0 (0.00)		
270 –	943	266 (28.21)			11 (4.14)		
Ever have five or more drinks every day			4.090	0.043		–	–
Yes	66	12 (18.18)			0 (0.00)		
No	328	100 (30.49)			2 (2.00)		
30 –	5	1 (20.00)			0 (0.00)		
90 –	6	2 (33.33)			0 (0.00)		
180 –	2	0 (0.00)			0 (0.00)		
270 –	718	203 (28.27)			11 (5.42)		
Vigorous activity past 30 days			–	–		3.279	0.070
Yes	298	78 (26.17)			0 (0.00)		
No	421	122 (28.98)			5 (4.10)		
Moderate activity past 30 days			–	–		3.039	0.081
Yes	367	106 (28.88)			1 (0.94)		
No	362	99 (27.35)			5 (5.05)		
Frequency of playing or exercising hard every week			–	–		3.841	0.050
< 7	92	27 (29.35)			2 (7.41)		
7 –	112	39 (34.82)			3 (7.69)		
15 –	4	0 (0.00)			0 (0.00)		
21 –	2	1 (50.00)			0 (0.00)		
30 –	800	219 (27.38)			6 (2.74)		
Number of hours using TV, video or computer everyday			–	–		9.332	0.002
None	5	0 (0.00)			0 (0.00)		
< 1	42	14 (33.33)			0 (0.00)		
1	47	9 (19.15)			1 (11.11)		
2	77	27 (35.06)			0 (0.00)		
3	63	15 (23.81)			0 (0.00)		
4	32	11 (34.38)			0 (0.00)		
> 5	67	24 (35.82)			0 (0.00)		

(0.00%), levofloxacin (0.00%), rifampin (0.00%), amoxicillin (0.00%), chloramphenicol (0.00%), doxycycline (0.00%), daptomycin (0.00%), quinupristin-dalfopristin (0.00%), and linezolid (0.00%). Specially, there was no MSSA strain resistant to tetracycline, vancomycin, clindamycin, imipenem, cefazolin, oxacillin, gentamicin, ciprofloxacin, levofloxacin, rifampin, amoxicillin, chloramphenicol, doxycycline, daptomycin, quinupristin-dalfopristin, and linezolid. Moreover, there was no MDR *S. aureus* strain. There were significantly differences between MRSA and MSSA strains in tetracycline (P = 0.012), imipenem (P = 0.007), cefazolin (P = 0.007), oxacillin (P < 0.001), levofloxacin (P = 0.007), and amoxicillin (P = 0.070). More details can be found in Table IV.

Toxin characteristics of *S. aureus* nasal carriage. No MRSA strain was positive to SEA while 28.57% of MSSA strains were positive to it. No MRSA strain was positive to SEB while 4.76% of MSSA strains were positive to it. 18.18% of MRSA strains were positive to SEC while 9.52% of MSSA strains were positive to it. 36.36% of MRSA strains were positive to SED while 4.76% of MSSA strains were positive to it. Both MRSA and MSSA strains were not positive to SEE or SEH. 9.09% of MRSA strains were positive to STTS-1 while 38.10%

Table II
Logistic regression analysis of *Staphylococcus aureus* nasal carriage

Influencing factors	OR	95% CI
Military status		
Yes	0.371	0.091–1.514
No	1.00	
Ever told you had cancer or malignancy		
Yes	0.255	0.051– 1.286
No	1.00	
Blood relatives have diabetes		
Yes	1.680	0.567–4.982
No	1.00	
Taken antibiotics past month		
Yes	0.552	0.100–3.045
No	1.00	
Take diabetic pills to lower blood sugar		
Yes	1.797	0.691–4.677
No	1.00	
Had a painful sensation or tingling in hands or feet past three months		
Yes	0.359	0.146–0.882
No	1.00	
Number of days used street drugs over past year		
< 30	1.00	
30 –	–	–
90 –	–	–
180 –	–	–
270 –	1.951	0.526–7.236
Ever have five or more drinks every day		
Yes	0.317	0.079–1.269
No	1.00	

OR, Odds Ratio; CI, confidence interval.

Table III
Logistic regression analysis of MRSA nasal carriage

Influencing factors	OR	95% CI
Gender		
Male	1.00	
Female	3.410	1.091–10.653
Frequency of play or exercise hard every week		
< 7	1.00	
7 –	0.976	0.148–6.427
15 –	–	–
21 –	–	–
30 –	0.323	0.061–1.722

OR, Odds Ratio; CI, confidence interval.

of MSSA strains were positive to it. With regard to PVL, 18.18% of MRSA strains were positive to it while no MSSA strain was positive to it. There were significantly differences between MRSA and MSSA strains in SEA (P = 0.049), SED (P = 0.019), TSST-1 (P = 0.083), and PVL (P = 0.044). More details can be found in Table V.

Molecular characteristics of MRSA nasal carriage. With regard to outcomes of SCC*mec* type among 11 MRSA strains, IV (63.64%) was the highest, followed by II (27.27), NT (9.09), I (0.00), III (0.00), and V (0.00). 63.64% of MRSA strains were CA, 27.27% were HA, and 9.09% were NT.

Discussion

This current study comprehensively elucidate the prevalence, influencing factors, antibiotic resistance, toxin and molecular characteristics of *S. aureus* and MRSA nasal carriage among diabetic population who participated in the NHANES, 2001–2004. Among 1010 diabetic population in this current study, there were 286 *S. aureus* strains, 11 MRSA strains, and 275 MSSA strains. The prevalence of *S. aureus*, MRSA, and MSSA nasal carriage were 28.32% (286/1010), 1.09% (11/1010), and 27.23% (275/1010) respectively. The prevalence of *S. aureus* (28.32%, 286/1010) nasal carriage among diabetic population in this current study was lower than that of diabetic outpatient population in Turkey (41.78%, 127/304) (Kutlu *et al.*, 2012), that of long-term hemodialysis type 2 diabetes patients in Saudi Arabia (72.41%, 42/58) (Saxena *et al.*, 2002), that of hospitalized diabetic patients in India (56.67%, 34/60) (Ahluwalia *et al.*, 2000), and that of community-based diabetes patients in Australia (39.09%, 258/660) (Hart *et al.*, 2015), but was higher than that of hospitalized diabetic patients (20.50%, 41/200) (Junhua *et al.*, 2005) and community-based type 2 diabetes patients in China (10.31%, 43/417) (Yan *et al.*, 2015). The prevalence of MRSA (1.09%, 11/1010) nasal carriage among *S. aureus* diabetic population in this current study was lower than community-based type 2 diabetes patients in China (5.28%, 22/417) (Yan *et al.*, 2015), that of diabetic outpatient population in Turkey (9.87%, 30/304) (Kutlu *et al.*, 2012), and that of long-term hemodialysis type 2 diabetes patients in Saudi Arabia (18.97%, 11/58) (Saxena *et al.*, 2002), but was higher than hospitalized diabetic patients in China (0.50%, 1/200) (Junhua *et al.*, 2005), that of community-based diabetes patients in Australia (1.21%, 8/660) (Hart *et al.*, 2015), and that of type 1 diabetes pediatric outpatients in Turkey (in 2005, 0.99%, 1/101; in 2013, 0.75%, 1/134) (Karadag-Oncel *et al.*, 2015). From the above statistics, we can know that the prevalence of *S. aureus* and MRSA nasal carriage among diabetic population were different in different countries and regions, and diabetic population might be more likely to carry *S. aureus* and MRSA in United States.

Table IV
Antibiotic resistance of *Staphylococcus aureus* nasal carriage

Antibiotic	MRSA		MSSA		χ^2	P
	Number of population	Resistant (%)	Number of population	Resistant (%)		
Tetracycline	11	3 (27.27)	21	0 (0.00)	6.320	0.012
Clindamycin	11	2 (18.18)	21	0 (0.00)	–	0.111
Erythromycin	11	8 (72.73)	21	19 (90.48)	1.725	0.189
Penicillin	11	11 (100.00)	21	19 (90.48)	–	0.534
Imipenem	2	2 (100.00)	15	0 (0.00)	–	0.007
Vancomycin	11	0 (0.00)	21	0 (0.00)	–	–
Cefazolin	2	2 (100.00)	15	0 (0.00)	–	0.007
Oxacillin	11	11 (100.00)	21	0 (0.00)	32.000	< 0.001
Gentamicin	11	0 (0.00)	21	0 (0.00)	–	–
Ciprofloxacin	2	0 (0.00)	15	0 (0.00)	–	–
Levofloxacin	11	3 (27.27)	21	0 (0.00)	6.320	0.012
Rifampin	11	0 (0.00)	21	0 (0.00)	–	–
Amoxicillin	2	2 (100.00)	15	0 (0.00)	–	0.007
Chloramphenicol	9	1 (11.11)	6	0 (0.00)	–	1.000
Doxycycline	2	0 (0.00)	1	0 (0.00)	–	–
Daptomycin	5	0 (0.00)	3	0 (0.00)	–	–
Quinupristin-dalfopristin	6	0 (0.00)	3	0 (0.00)	–	–
Linezolid	9	0 (0.00)	6	0 (0.00)	–	–

Table V
Toxin characteristics of *Staphylococcus aureus* nasal carriage [n (%)]

Toxin	Total (N = 32)	MRSA (N = 11)	MSSA (N = 21)	χ^2	P
SEA	6 (18.75)	0 (0.00)	6 (28.57)	3.868	0.049
SEB	1 (3.13)	0 (0.00)	1 (4.76)	–	1.000
SEC	4 (12.50)	2 (18.18)	2 (9.52)	0.495	0.482
SED	5 (15.63)	4 (36.36)	1 (4.76)	5.468	0.019
SEE	0 (0.00)	0 (0.00)	0 (0.00)	–	–
SEH	0 (0.00)	0 (0.00)	0 (0.00)	–	–
TSST-1	9 (28.13)	1 (9.09)	8 (38.10)	3.004	0.083
PVL	2 (6.25)	2 (18.18)	0 (0.00)	4.073	0.044

SE, staphylococcal enterotoxin; TSST-1, toxic-shock syndrome toxin-1; PVL, panton valentine leukocidin; N, number of population; n, number of positive strains.

There were studies reported that the main influencing factors of *S. aureus* and MRSA nasal carriage were gender (Geofrey *et al.*, 2015; Soltani *et al.*, 2014; Yan *et al.*, 2015), age (Geofrey *et al.*, 2015; Huifen *et al.*, 2015; Soltani *et al.*, 2014), weight (Campbell *et al.*, 2015), history of hospitalization (Huifen *et al.*, 2015; Karanika *et al.*, 2015), history of invasive operation (Karanika *et al.*, 2015; Qiongxiang, 2014), conditions of antibiotic using (Qiongxiang, 2014; Soltani *et al.*, 2014), chronic diseases (Campbell *et al.*, 2015; Chen and Pass, 2013; Daeschlein *et al.*, 2015; Geofrey *et al.*, 2015; Huifen *et al.*, 2015; Karanika *et al.*, 2015), history of infection (Chen and Pass, 2013; Daeschlein *et al.*, 2015) and so on. After performing a logistic regression, ever had a painful sensation or tingling in hands or feet past three months was still associated with *S. aureus* nasal carriage among diabetic population in this current study. Having a painful sensation or tingling in hands or feet past three months was a protective factor, which might be resulted from more likely of this population to notice the hygiene of their hands or feet. We found that having a painful sensation or tingling in hands or feet past three months among diabetic population was 0.359 times less likely than those not having a painful sensation or tingling in hands or feet past three months to carry *S. aureus*. With regard to

MRSA nasal carriage among diabetic population in this current study, gender was still associated with it in this logistic regression model. Female was a risk factor, which might be resulted from their weaker immune system. Female diabetic population was 3.410 times more likely than male diabetic population to carry MRSA, which was consistent to a Chinese study (Yan *et al.*, 2015) and a Tanzanian study (Soltani *et al.*, 2014). Therefore, female diabetic population should be paid more attention to perform surveillance of *S. aureus* and MRSA so as to avoid carriage and/or infection.

We found that both MRSA and MSSA strains in this current study were highly resistant to erythromycin and penicillin, which was similar to several studies (Geofrey *et al.*, 2015; Junhua *et al.*, 2005; Kejela and Bacha, 2013; Morgenstern *et al.*, 2016) and this might be resulted from the extensive use of these antibiotics in medical institutions. But both MRSA and MSSA strains were 100% sensitive to vancomycin, gentamicin, ciprofloxacin, rifampin, doxycycline, daptomycin, quinupristin-dalfopristin and linezolid, which was similar to several studies (Junhua *et al.*, 2005; Morgenstern *et al.*, 2016), and it could indicate that these antibiotics can be used to treat *S. aureus* infection in clinical medication. The propotions of resistance to tetracycline ($P = 0.012$), imipenem ($P = 0.007$), cefazolin ($P = 0.007$), oxacillin ($P < 0.001$), levofloxacin ($P = 0.007$), and amoxicillin ($P = 0.070$) among MRSA strains were significantly higher than MSSA strains. This could indicate that the antibiotic resistance of MRSA strains was more severe than MSSA strains and should be emphasized by healthcare workers. It suggested that healthcare workers should rationally utilize antibiotics, prevent antibiotic abuse and long-term use, and can use antibiotic combination or rotation to avoid reducing sensitivity of *S. aureus* and MRSA to antibiotics.

The pathogenesis of *S. aureus* infections depends on the production of surface proteins that mediate bacterial adherence to host tissues, secretion of a series of extracellular toxins, and enzymes that destruct host cells and tissues, avoidance of, or incapacitating, the host immune defense, and growth and spread of bacteria in host cells (Lowy, 1998). Toxins are proteins secreted by *S. aureus* into the extracellular matrix during the post-exponential and early stationary phases. These proteins are usually involved in tissue penetration and enable the bacteria to invade its host. They are also cytolytic and help bacterial growth by acquiring essential nutrients such as iron from lysed-cells (Kong *et al.*, 2016). The SEs genes are super antigens which trigger T-cell activation and proliferation, and their mode of action probably includes activation of cytokine release and cell death *via* apoptosis and potentially lethal toxic shock syndrome (Lin *et al.*, 2010). In this current study, 18.18% and 36.36% of MRSA strains were positive to SEC and SED, respectively. 28.57%, 4.76%, 9.52%, and 4.76% of MSSA strains were positive to SEA, SEB, SEC, and SED, respectively. Because SEs can cause vomiting and diarrhea and the toxins are one of the most common causes of food-borne diseases (Kong *et al.*, 2016), we should pay more attention to those who were positive to them. It has been reported that TSST-1 will stimulate the release of chemokines, such as IL-8 and MIP-3α, IL-2, and TNFα (Otto, 2014). Activation of immune cells will enhance inflammation and cause mucosal cell barrier disruption, allowing further interaction of the toxin with T-cells and macrophages, leading towards toxic shock syndrome (Larkin *et al.*, 1982). MSSA (38.10%) strains were more likely than MRSA (9.09%) strains to be positive to TSST-1 ($\chi^2 = 3.004$, $P = 0.083$), which meant that those who carried MSSA should be paid more attention. PVL has been reported to be associated with CA-MRSA infections (Otto, 2010), which was consistent with this current study. 28.57% (2/7) of CA-MRSA strains in this current study were positive to it. 18.18% of MRSA strains were positive to PVL, which was higher than health care personnel in Egypt (2.24%, 5/223) (Hefzy *et al.*, 2016) and patients in Indonesia (6.56%, 17/259) (Santosaningsih *et al.*, 2016), which might be resulted from poor hand hygiene compliance and non-judicious use of antibiotics.

Among 11 MRSA strains, seven (63.63%) were CA, three (27.27%) were HA, and one (9.09%) was NT, which could indicate that the majority of MRSA strains in this current study were from community and relevant departments should pay attention to strengthen the surveillance and disinfection in community.

Overall, there were several limitations in this current study. Firstly, we did not take the environmental characteristics into consideration due to the complex data due to limited financial support. Secondly, we did not follow up the outcome of the included population due to the limited data, which can be studied in the future. Thirdly, there are limited data on the antibiotic resistance, toxin, and molecular characteristics of *S. aureus* and MRSA, which might lead to some kind of errors.

Conclusions

Diabetic population might be more likely to carry *S. aureus* and MRSA in United States. Having a painful sensation or tingling in hands or feet past three months might be a protective factor. Female might be a risk factor and female diabetic population should be paid more attention to perform surveillance of *S. aureus* and MRSA so as to avoid carriage and/or infection. The antibiotic resistance of MRSA strains was more severe than that of MSSA strains and should be emphasized by health-

care workers. The proportions of toxins in *S. aureus* were high and those who detected positive should be paid more attention. Therefore, improving compliance, reducing antibiotic overuse, screening for carriers, and decolonization are recommended strategies for reducing the spread of *S. aureus* and MRSA. The majority of MRSA strains in this current study were from community and relevant departments should pay greater attention to strengthen the surveillance of *S. aureus* and MRSA and disinfection measures in community.

Supplementary Materials

Supplementary materials were tables used in the study for the univariate results of influencing factors among *S. aureus* and MRSA nasal carriage. Supplementary materials accompanies the paper on Polish Journal of Microbiology website.

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Conflict of interest

The authors declare that they have no conflict of interest.

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ORIGINAL PAPER

Production, Characterization and Valuable Applications of Exopolysaccharides from Marine *Bacillus subtilis* SH1

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Abstract

Exopolysaccharides (EPSs) are high molecular weight polymers consisting of different sugar residues they are preferable for replacing synthetic polymers as they are degradable and nontoxic. Many microorganisms possess the ability to synthesize and excrete exopolysaccharides with novel chemical compositions, properties and structures to have potential applications in different fields. The present study attempt to optimize the production of EPS by marine *Bacillus subtilis* SH1 in addition to characterization and investigation of different valuable applications. Effect of medium type, incubation period and pH were studied using the one factor at a time experiments. It was shown that the highest productivity (24 gl^{-1}) of exopolysaccharides was recorded by using yeast malt glucose medium with pH 9 at the fourth day of incubation. Experimental design using Response Surface Methodology (RSM) was applied to optimize various nutrients at different concentrations. The finalized optimized medium contained (gl^{-1}) glucose (5), peptone (2.5), yeast extract (4.5) and malt extract (4.5) increased the production of EPS to 33.8 gl^{-1} with1.4 fold increase compared to the basal medium. Chemical characterization of the extracted EPS showed that, FTIR spectra exhibited bands at various regions. Moreover, HPLC chromatogram indicated that the EPS was a heteropolysaccharide consisting of maltose and rhamnose. The study was extended to evaluate the potentiality of the extracted polysaccharides in different medical applications. Results concluded that, EPS exhibited antibacterial activity against *Aeromonas hydrophila*, *Pseudomonas aeruginosa* and *Streptococcus faecalis* and the highest antibacterial activity (7.8, 9 and 10.4 AU/ml) was against *S. faecalis* at 50, 100 and 200 mg/ml respectively. The EPS exhibited various degree of antitumor effect toward the tested cell lines (MCF-7, HCT-116 and HepG2). In addition, EPS exhibited antiviral activity at 500 µg/ml. The antioxidant capacity increased with increasing the concentration of the sample. Scanning electron microscopic analysis showed that EPS had compact film-like structure, which could make it a useful in the future applications as in preparing plasticized film.

Key words: *Bacillus subtilis* SH1, anticancer, antiviral, exopolysaccharides, response surface methodology

Introduction

Exopolysaccharides (EPSs) are defined as; high molecular weight, biodegradable polymers which biosynthesized by a wide range of organisms (Vijayabaskar *et al*., 2011; Sanlibaba and Çakmak, 2016). The EPS are often found in the surroundings of the outer structures of prokaryotic as well as eukaryotic microbial cells. They are either closely associated with the cell in the form of discrete capsules or else are excreted as slime unattached to the cellular surface. They exist in a wide variety of unique and complex chemical structures and are believed to provide self-protection against antimicrobial substances growing nearby (Nanda and Raghavan, 2014). Exopolysaccharides have major roles in different processes viz., formation of biofilm (Ohno *et al*., 2000; Vimala and Lalithakumari, 2003), protection of bacterial cell from desiccation, for maintaining primary cellular functions and antibacterial activity against predators, gelling ability, pollutant degradation kinetics and cement based construction industry (Bhaskar and Bhosle, 2005; Rawal *et al*., 2016). There are two major types of EPSs according to the constituents; homo- and hetero-EPSs. Recently, microbial polysaccharides have been attended due to their unique properties and the possibility of quick mass production. Today, EPSs find wide range of applications in food, pharmaceutical, other industries (Patil *et al*., 2009; Nwodo *et al*., 2012). Also, they have proved various physiological activities in human beings as anti-tumor, anti-viral and anti-inflammation agents, as well as being inducers for interferon, platelet aggregation inhibition, colony stimulating factor synthesis, coagulants and lubricants (de Godoi *et al*., 2014; Li *et al*., 2016; Venkateswarulu *et al*., 2016). A number of microbial strains have the potentiality to produce polysaccharides with widely varying compositions. On the other

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side, EPSs production from marine microorganisms such as; *Zoogloea* sp. (Ikeda *et al*., 1982), *Pseudomonas* sp. (Matsuda and Worawattanamateekul, 1993), *Vibrio fischeri* (Rodrigues and Bhosle, 1991), *Cyanothece* sp. (Philippis *et al*., 1993), and *Alteromonas macleodii* (Raguenes *et al*., 1996) were reported and their properties were studied well. Furthermore, *Bacillus* sp. produces complex EPSs and widely studied species were; *Bacillus licheniformis*, producing levan (Ghaly *et al*., 2007), *Bacillus coagulans*, *Bacillus polymyxa* (Lee *et al*., 1997) and *Bacillus mucilaginous* (Lian *et al*., 2008). *Bacillus subtilis* is also one of the major producers of EPS among *Bacillus* sp. In major reports, *B. subtilis* is mostly found to produce a biopolymer poly-γ-Glutamate (PGA) (Jane and HsiuFeng, 2007; Robert *et al*., 2011), levan, fructan (De Melo *et al*., 2010). The optimization process of fermentation is very critical and parameters affecting could be carried out using a statistical tool; Response Surface Methodology (RSM). This method has many advantages over the conventional method which involves numerous experiments by changing one variable at a time with keeping other independent variable constant. The RSM is a reliable tool and a fast experimentation technique which deals with individual effects of nutrients and their interactive effects (Wu *et al*., 2008; Rabha *et al*., 2012; Prathima *et al*., 2014).

The main goal of the present study was the production, maximization and characterization of EPSs from the marine *B. subtilis* SH1. Moreover, different medical applications were investigated.

Experimental

Materials and Methods

Microorganism and culture conditions. Marine *B. subtilis* SH1 was kindly provided by Dr. Hassan A.H. Ibrahim; Marine Microbiology Dep., NIOF, Alexandria, Egypt. It was isolated upon seawater agar medium from Suez Gulf and completely identified by phenotypic and genotypic means. The nucleotide sequence was deposited to GenBank sequence database and has EU107759 accession number.

Reference bacterial strains. The bacterial indicator strains (*Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Vibrio damsela*, *Escherichia coli*, and *Streptococcus faecalis*) were kindly provided by Marine Microbiology Dep, NIOF, Alexandria, Egypt.

Production of EPS. *B. subtilis* SH1 was inoculated in 50 ml aliquot of nutrient broth medium dispensed in 250 ml Erlenmeyer flask, the flask was incubated at 37°C under shake condition (120 rpm) for overnight. After overnight incubation, 500 µl was transferred to 50 ml of a fresh production media in a conical flask. Above step was carried out in an aseptic manner, the flask was incubated for 4 days at 37°C.

Extraction of EPS from *B. subtilis* SH1 culture. The marine *B. subtilis* SH1 culture was centrifuged at 10,000 g for 10 min at 4°C. The supernatant obtained was mixed with two volumes of ice cold ethanol and kept at 4°C for 24 h. The mixture was then centrifuged at 2500 × g for 20 min at 4°C. The obtained pellet was suspended in distilled water, which was centrifuged at 2500 × g for 30 min at 4°C with two volumes of ice cold ethanol. The process was repeated twice and the EPS obtained was dried, weighed and lyophilized (Savadogo *et al*., 2004).

One factor at time experiments

Effect of different media on EPS production. To study the effect of media on the production of EPS, different media (gl^{-1}) including nutrient broth (Abou-Dobara *et al*., 2014); yeast extract 2, peptone 5, NaCl, 5 (NB) (Abou-Dobara *et al*., 2014), soft brown sugar 40; $MgSO_4 \cdot 7H_2O$ 0.2; K_2HPO_4 9; KH_2PO_4 3; yeast extract 2 (SEM) (Tallgren *et al*., 1999); yeast extract 3, malt extract 3, glucose 10, peptone 5 (YMG) (Abou-Dobara *et al*., 2014); Yeast mannitol (YM), yeast extract 0.5, mannitol 4 (Mukherjee *et al*., 2011) were tested. A liquots of 1% (OD = 1 at 600 nm) of the active culture of the *B. subtilis* SH1 which was previously proliferated in marine nutrient broth, was used as constant inoculum size for each medium.

Effect of different incubation period on EPS production. The effect of incubation time was investigated by varying time periods (1–6) days. Culture with 1% was used as constant inoculum size.

Effect of different pH on EPS production. The initial pH for higher product yield was determined by adjusting pH with 1M HCl and 1M NaOH before sterilization at different pH as 5, 6, 7, 8, and 9. Culture with 1% (OD = 1 at 600 nm) was used as constant inoculum size.

Optimization of culture conditions using RSM. Response surface methodology was used to determine the optimum concentration of the tested variables for enhancement of EPSs production. The variables were (glucose, peptone, yeast extract and malt extract). The four independent variables were evaluated at five different levels (–2, –1, 0, +1 and +2) and 30 experiments, containing 6 replications at the center point as shown in Table I. The behavior of the system was explained by the following second order polynomial equation:

$$Y = \beta 0 + \beta 1X1 + \beta 2X2 + \beta 3X3 + \beta 4X4 + \beta 12X1X2 + \\ + \beta 13X1X3 + \beta 14X1X4 + \beta 23X2X3 + \beta 24X2X4 + \\ + \beta 34X3X4 + \beta 11X12 + \beta 22\ X22 + \beta 33X32 + \beta 44X42$$

Where, Y is the predicted response, β0 is the scaling constant, X1-X4 are the coded levels of the fac-

Table I
Central composite design and the EPSs production by *B. subtilis* SH1 obtained from the culture trials

Run order	Coded levels				Response		
	X1 (Glucose)	X2 (Peptone)	X3 (Yeast extract)	X4 (Malt extract)	EPS (gL^{-1})		Biomass (gL^{-1})
					Observed values	Predicted values	
1	1	−1	−1	1	24.6	22.8	1.8
2	0	0	0	0	17.6	22.8	1.8
3	0	2	0	0	12.8	21	1.8
4	0	0	0	0	20	17.6	2
5	1	1	1	−1	9.6	21	2.6
6	−1	−1	1	1	33.8	5.8	1.6
7	0	0	0	−2	21.6	25	3.2
8	0	0	0	2	21.2	18	2.4
9	0	0	0	0	24	1.8	1.4
10	1	1	−1	1	31.8	21	2
11	−1	−1	−1	−1	14.4	36.8	1.4
12	−1	1	1	1	29.8	18	2
13	1	−1	1	−1	6.2	36	0.6
14	0	0	2	0	17.8	5.8	1.6
15	−2	0	0	0	33	17.8	1.6
16	0	0	0	0	12	28.6	2.4
17	−1	1	−1	1	23	21	1.2
18	1	1	−1	−1	20.8	20.6	2
19	0	0	0	0	22	26.8	2.2
20	0	0	0	0	20	21	2.4
21	2	0	0	0	24.8	21	2.4
22	0	−2	0	0	24.8	25	1.8
23	−1	1	−1	−1	25.6	21.6	1.4
24	−1	−1	1	−1	15.6	23	1.8
25	1	−1	1	1	16	11.2	1.6
26	−1	−1	−1	1	10.4	10.4	0.4
27	0	0	−2	0	27.4	23.6	1.6
28	−1	1	1	−1	27.4	26.2	2.2
29	1	−1	−1	−1	26.6	26.8	1.8
30	1	1	1	1	24.4	18	1.8

tors, β1-β4 are the linear coefficients, β12-β34 are the interactive coefficients and β11-β44 are the quadratic coefficients. ANOVA and regression analyses were also carried out used the above software. The quality of the polynomial equation was confirmed by the determination of coefficient R^2 and its statistical significance was determined by Fisher's test, F value.

Morphological characterization of EPS. Morphology of dried EPSs was determined by scanning electron microscopy examination (SEM). EPS was coated on gold particles and microstructure was visualized under scanning electron micrograph at 3000X. Whole and surface view of EPSs was taken and structure was analyzed (Yada *et al*., 2011).

Structural analyses. Pellets for infrared analysis were prepared by grinding a mixture of 2 mg polysaccharide with 200 mg dry KBr, followed by pressing the mixture into a 16 mm diameter mold. The Fourier transform-infrared (FTIR) spectra were recorded on a Bruker Vector 22 instrument with a resolution of 4 cm^{-1} in the 4000–400 cm^{-1} region. Chemical characterization included UV was carried out using UV spectroscopy (Heλiosα).

Composition of EPSs produced by marine *B. subtilis* SH1 was done using HPLC system. The EPS isolated from culture was dissolved in distilled water and EPS (1 ml) was hydrolyzed with 3 M Tri-fluoroacetic acid (TFA) for 1–2 h at 250°C using oil bath.

Hydrolyzed EPS was neutralized using 5 N NaOH and volume was made to 50 ml with MilliQ water. HPLC of hydrolyzed EPSs was done by using aminopropile column (HI-Plex, Agilent, 4.6 × 250 mm), acetonitrile and water was used as mobile phase in ratio of 75:25. The separation was carried out at 24°C with flow rate of 1 ml/min and sample volume of 20 µl. Monosaccharides such as glucose, arabinose, raffinose, rhamnose, maltose, *etc*. were used as standard. The column was calibrated with different molecular mass standard and a standard curve was then established (Yada *et al*., 2011).

Antimicrobial activity of EPS against indicator strains. Fifty milliliter of nutrient agar medium inoculated with indicator pathogenic bacteria (1% v/v) were poured into all plates. After solidifying, wells were punched out using 0.5 cm cork borer, and each of their bottoms was then sealed with two drops of sterile water agar. One hundred microliters of different concentrations (10, 25, 50, 100 and 200 mg/ml) of filtrated EPS were transferred into each well after sterilizing by ultrafiltration using 0.22 µm sterilized filters. All plates were incubated at appropriate temperature for 24–48 h. After incubation period, the radius of clear zone around each well (Y) and the radius of the well (X) were linearly measured in mm, where dividing Y^2 over X^2 determines an absolute unit (AU) for the clear zone. The absolute unit of each EPS, which indicates a positive result in the antimicrobial action, was calculated according to the following equation: $AU = Y^2/X^2$ (El-Masry *et al*., 2002).

Antitumor activity. Human Breast cancer (MCF-7), Human colon carcinoma HCT-116 and Hepatocellular carcinoma (HepG2) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were grown on RPMI-1640 medium supplemented with 10% v/v inactive fetal calf serum and 50 µg/ml Gentamycin. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and were subcultured two to three times a week.

The antitumor activity was evaluated on tumor cells. The cells were grown as monolayers in growth medium supplemented with 10% inactive fetal calf serum and 50 µg/ml Gentamycin. The monolayers of 10000 cells adhered at the bottom of the wells in a 96-well microliter plate incubated for 24 h at 37°C in a humidified incubator with 5% CO_2. The monolayers were then washed with sterile phosphate buffered saline (0.01 M, pH 7.2) and simultaneously the cells were treated with 100 µl from different dilutions of tested sample in fresh maintenance medium and incubated at 37°C. A control of untreated cells was made in the absence of tested sample. Six wells were used for each concentration of the test sample. Every 24 h the observation under the inverted microscope was made. The number of the surviving cells was determined by staining the cells with crystal violet (Gangadevi and Muthumary, 2007) followed by cell lysing using 33% glacial acetic acid and read the absorbance at 590 nm using ELISA reader (SunRise, TECAN, Inc, USA) after well mixing. The absorbance values from untreated cells were considered a 100% proliferation.

The number of viable cells was determined using ELISA reader as previously mentioned before and the percentage of viability was calculated as (ODt/ODc) × 100% where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells. The 50% inhibitory concentration (IC50), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots.

Antiviral assay

Mammalian cell line. Vero cells (derived from the kidney of normal African green monkeys) were obtained from agle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, HEPES buffer and Gentamycin (50 µg/ml). All cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and were subcultured two times a week.

Evaluation of the antiviral activity using cytopathic inhibition assay. The screening of antiviral assay using cytopathic effect inhibition was carried out at the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University. This assay was selected to show specific inhibition of a biologic function (Hu and Hsiung, 1989). Monolayers of 1000 vero cells adhered at the bottom of the wells in a 96-well microliter plate incubated at 24 h at 37°C in a humidified incubator with 5% CO_2. The plates were washed with fresh DMEM and challenged with 10^4 herpes simplex type I virus (HSV-1) doses and simultaneously the cultures were treated with two-fold serial dilutions of tested compound in fresh maintenance medium and incubated at 37°C for 3 days. An infection treatments as well as untreated vero cells control was made in the absence of tested compound. Six wells were used for each concentration of the tested compound. Every 24 h the observation under the inverted microscope was made until the virus in the control wells showed complete viral- induce cytopathic effects. Antiviral activity was determined by the inhibition of cytopathic effect compared to control (Vijayan *et al*., 2004).

Three independent experiments were assessed each containing four replicates per treatment. Acyclovir, which is clinically used for the treatment of hepatic viral disease, was used as a positive control under this assay system (Dargan, 1998).

After the incubation period, the media was aspirated, and the cells were stained with a 0.1% crystal violet solution for 4 h. The stain was removed and the plates rinsed using tap water until all excess stain was

removed. The plates were allowed to dry for 24 h and then glacial acetic acid (30%) was then added to all wells and mixed thoroughly, and then the absorbance of the plates were measured after gently shaken on Microplate reader (TECAN, Inc) at 620 nm. Viral Inhibition rate was calculated as follows:
[(ODtv-ODcd/ODcv)] × 100%, where ODtv, ODcv and ODcd indicate the absorbance of the test compounds with virus infected cells, the absorbance of the virus control and the absorbance of the cell control, respectively.

Data and statistical analysis. From these data, the dose that inhibited viral infection by 50% (EC50) was estimated with respect to virus control from the graphic plots using STATA modeling software. EC50, the effective concentration needed to restrain 50% virus infection compared to untreated infected cells, was determined directly from the curve obtained by plotting the inhibition of the virus yield against the concentration of the samples. To determine if each compound has sufficient antiviral activity that exceeds its level of toxicity, a selectively index (SI) was calculated. The selectivity index (SI) was measured from the ratio of the concentration at which 50% cytotoxicity was observed (CC50/EC50) (Zandi *et al.*, 2007). This index, also referred to as a therapeutic index, was used to determine if a compound warrants further study. Compounds that had an SI of 2 were considered active, 10 or greater was considered very active.

Antioxidant activity using DPPH stable free radical scavenging assay. The DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) stable free radical scavenging assay was performed as previously described by Hu and Kitts, 2000 with modifications. Sample was dissolved in a methanol at different concentrations ranging from 1 to 128 µg/ml and then of the sample (2 ml) was incubated with 0.5 ml (0.2 mM) DPPH in 100% methanol. Sample absorbance at 519 nm was recorded after 30 min of incubation at room temperature. Inhibition of the DPPH stable free radical was calculated as follows:

$$\text{Inhibition } \% = (Ab_{Control} - Ab_{Sample})/(Ab_{Control} - Ab_{Blank}) \times 100$$

Where:

Ab control = absorbance of 0.1 mM DPPH alone in methanol;
Ab sample = absorbance of 0.1 mM DPPH + sample in methanol; and
Ab blank = absorbance of methanol solvent control in absence of DPPH or sample.

Results and Discussion

B. subtilis is one of the major producers of exopolysaccharides (EPSs) among *Bacillus* sp. Therefore, the current study was suggested to evaluate the production and characterization of EPSs from marine *B. subtilis* SH1, as well as, to detect some medical applications of EPSs.

Optimization of fermentation conditions for EPS production by marine *B. subtilis* SH1

Effect of different media on EPS production. Different media including NB, SEM, YMG and YM were evaluated for the production of EPS by *B. subtilis*. As shown in Fig. 1, YMG medium was the most suitable for higher production of EPS with yield of 18 gl^{-1} followed by NB (11.2 gl^{-1}) and YM (8.8 gl^{-1}), while the lowest production (8.4 gl^{-1}) was recorded up on using SEM medium. Similar results were recorded by Abou-Dobara *et al.* (2014), who stated that among seven tested media, YMG medium gave the highest production of EPS by *B. subtilis*. They also reported that glucose, malt and mannitol support good biomass production.

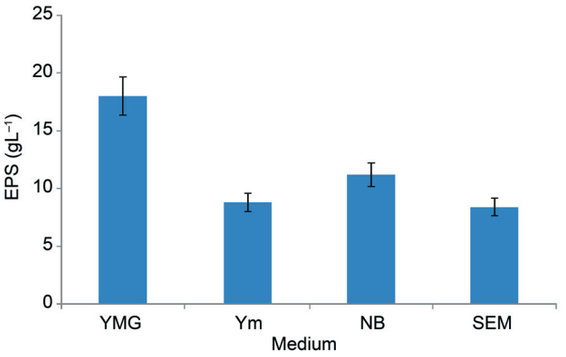

Fig. 1. Effect of different media on the production of the EPSs by marine *B. subtilis* SH1.

Effect of incubation time on the production of EPS by *B. subtilis* SH1. Incubation time is an essential factor determining the enhancement of EPS synthesis in the culture. Observation of time course of EPS production in association with growth of *B. subtilis* SH1 at different time interval (1–6 days) (Fig. 2) indicated that the production of EPS increased gradually from 17.2 gl^{-1} at the first day with biomass of 20 gl^{-1} to 19.8 gl^{-1} at the fourth day which represented the highest productivity of EPS and also the highest biomass production (44 gl^{-1}), while the production decreased after that till the last day to reach 18 gl^{-1}. This can be explained by the fact that EPS is highly synthesized during late exponential growth phase and decrease in incubation time may lower the production. Higher incubation time might affect the yield due to the production of certain enzymes, saccharases, along with EPS, might act upon polysaccharides, thus deteriorating the product formation. The result of the present work is consistent with similar studies carried out earlier which reported the production of

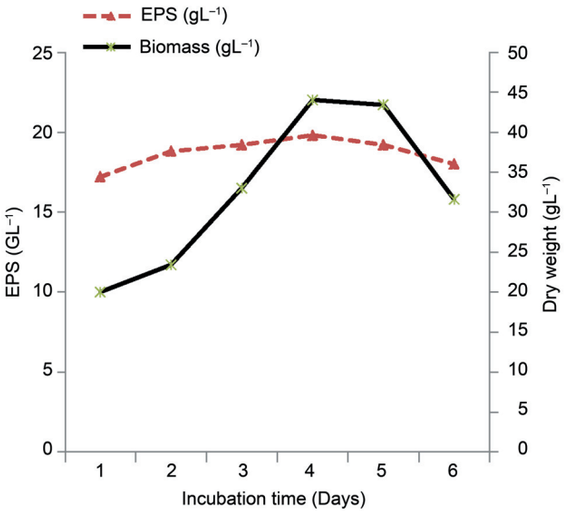

Fig. 2. Effect of incubation period on growth and EPSs production by marine *B. subtilis* SH1.

EPS by *B. subtilis* at late phase of growth (72 h) (Lee *et al.*, 1997; Vijayabaskar *et al.*, 2011).

Effect of pH on EPS production by *B. subtilis* SH1. The pH is a significant factor influencing the physiology of a microorganism by affecting nutrient solubility and uptake, enzyme activity, cell membrane morphology, by product formation and oxidative-reductive reactions (Bajaj *et al.*, 2009). Production of EPS by *B. subtilis* SH1 was estimated at different pH (5–9). As shown in Fig. 3, the production of EPS increased from 19 gl^{-1} at pH 5 to 19.8 gl^{-1} at pH 7 and the highest productivity of EPS was recorded at pH 9 which realized 24 gl^{-1} which means that the production was favorable at the alkaline level. These results coincide with those reported by Ko *et al.* (2000), who reported that the highest production of EPS by *Bacillus* sp. was produced at pH 8 and reduced in lower pH values. Relatively low concentration of EPS produced by *B. subtilis* was obtained in other studies at pH 7 and reduced at lower pH values (Abdul Razack *et al.*, 2013). Also, other studies reported that the maximum production of EPS by *B. subtilis* was obtained at pH 7 and decreased gradually at pH 8 to10 (Abu-Dobara *et al.*, 2014).

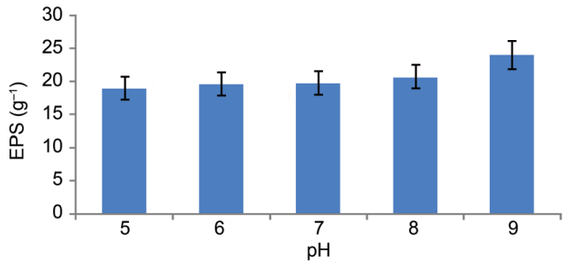

Fig. 3. Effect of different pH on the EPSs production by marine *B. subtilis* SH1.

Optimization of EPS production by *B. subtilis* SH1 using Response Surface Methodology (RSM). Response Surface Methodology (RSM) was used to investigate the influence of different components on the yields of biomass and extracellular EPS by *B. subtilis* SH1. Central composite design with 4 factors at five levels was used for fitting data on a second-order polynomial model (Table II). The mathematical method describing the relationships between the process responses (the yield of EPS) and the medium contents was developed. Different trials and the response were illustrated in Table I.

Table II
Levels of process variables used in central composite design.

Variables (gL^{-1})	Coded levels				
	−2	−1	0	1	2
Glucose (X1)	2.5	5	10	15	20
Peptone (X2)	1.25	2.5	5	7.5	10
Yeast extract (X3)	0.75	1.5	3	4.5	6
Malt extract (X4)	0.75	1.5	3	4.5	6

Results in Table I showed that the EPS yield varied between 6.2–33.8 gl^{-1}. The maximum productivity of EPS was achieved at conditions of low concentrations of glucose and peptone (5 g and 2.5 g) respectively but with high concentrations of yeast extract and malt extract (4.5 gl^{-1}). In agreement with the present study, Looijesteijn *et al.* (2000) studied the influence of different substrate limitations on EPS production by closely related organism namely *Lactococccus lactis* subsp. *cremoris*. They observed that reduction in glucose resulted in slightly higher production of EPS.

Kimmel *et al.* (1998) reported that higher glucose concentration (Carbon source) provides the higher yield of EPS for *L. lactis* sp. Abou-Dobara *et al.* (2014) reported that the highest production of EPS was recorded by using yeast extract with a concentration of 0.22%. Conversely, reports suggested that nitrogen limitation and higher amounts of carbon in the medium could yield a maximum amount of EPS. A study showed that EPS production from *Rhizobium meliloti* was higher when the nitrogen source was in minimal quantity. Similarly, pullulan was generated by *Aureobasidium pullulans* when it was grown in a medium with lesser amounts of nitrogen source. On the other hand, EPS production was observed under nitrogen-limited conditions (Mengistu *et al.*, 1994; Marshall *et al.*, 1995). It was observed that the growth culture conditions that realized the highest EPS production do not realize the highest growth. It was stated that biosynthesis of biomass and EPS biosynthesis follow roughly the same metabolic pathways. This results in the same meta-

bolic control for EPS production and for growth. At higher growth rates, more intermediates per time unit are needed for the biosynthesis of cell surface polysaccharides, and the intermediates are apparently used in favor of the synthesis of these polysaccharides; this may explain the reduction of the EPS yields at higher growth rates (Prathima *et al*., 2014).

It can be concluded that the present study achieved good yield of EPS (33.8 gl^{-1}) using the marine *B. subtilis* SH1 under the optimized conditions which is more yield in shorter time than other previous studies such as Shukla *et al*. (2015), who reported that the highest production of EPS was 12 gl^{-1}at 144 h. Also, Berekaa (2014) stated that the highest yield of EPS by *B. licheniformis* strain-QS5 was 22.5 gl^{-1}at 96 h under the optimized conditions. Thus the marine *B. subtilis* SH1 is promising candidate for EPS production as it is cheap and ecofriendly marine source.

Based on the results of the experimental designs, a second order polynomial equation was developed, describing the correlation between the variables used for study. The EPS yield could be represented as:

$$Y = 8.40 + 0.553\ X1 + 3.738\ X2 + 4.510\ X3 - 4.191\ X4 + \\ + 0.081\ X21 - 0.341 \times 22 - 0.0001\ X23 - 0.261 \times 24 - \\ - 0.065\ X1\ X2 - 0.868\ X1X3 + 0.118\ X1 \times 4 - \\ - 0.003\ X2X3 + 0.450X2X4 + 1.039\ X3X4.$$

Where, Y is the response *i.e*. EPS; X1, X2, X3 and X4 are the uncoded values of the test variables peptone, glucose, yeast extract and malt extract. Regression equation for the levels of EPS production (Y) as functions of; glucose concentration (X1), peptone concentration (X2), yeast extract (X3), and malt extract (X4) suggested that all the four factors influenced the EPS production by such organism.

ANOVA and regression analysis done for the experimental designs are tabulated (Table III). There was a significant difference ($p < 0.05$) and the second order model showed fit with $R^2 = 0.8$.

Table III
Statistical analysis of RSM.

Terms	EPS
F-value	1.67
P > F	0.167
R^2	0.8
Lack of fit	10
Pure error	5

Three dimensional response surface plots represent regression equations and illustrate interaction between the response and experimental levels of each variable was illustrated as shown in Fig. 4a–4f.

The results showed that the EPS yield varied between 6.2–33.8 gl^{-1}. The maximum productivity of EPS was achieved at concentrations (gl^{-1}): glucose, 5; peptone, 2.5; yeast extract, 4.5 and malt extract, 4.5. The production decreased under other conditions, irrespective of higher or lower than these levels.

These figures showed that the highest yield of EPS was recorded upon decreasing the concentrations of glucose and peptone (Fig. 4a) or decreasing the concentration of glucose and increasing the concentration of yeast extract (Fig. 4b). The same will be realized at low glucose concentration and high concentration of malt extract (Fig. 4c). Our finding is in accordance with Prathima *et al*. (2014), who reported the production of EPS by *L. lactis* NCDC 191 in a whey based medium under glucose limitation using Response Surface Methodology.

Results in Fig. 4d showed that interaction between low concentration of peptone and high concentration of yeast extract cause increase in the EPS yield, which also can be achieved in case of low concentration of peptone and high concentration of malt extract (Fig. 4e). On the other hand, increasing concentrations of both yeast extract and malt extract achieved the highest yield of EPS (Fig. 4f).

Morphological characterization of EPS. Textural and rheological properties of EPS are contributed by its structure beside its quantity and composition (Yada *et al*., 2011). The EPS obtained from *B. subtilis* SH1 was observed by SEM (Fig. 5). As it is seen from the microstructure of EPS surface view, the produced EPS is highly compact in structure. This indicates the potential of EPS as viscosifying, as a thickener or as stabilizing agent for novel food products (Yada *et al*., 2011).

Structural analyses. The FTIR spectra of the EPS (Fig. 6) exhibited bands at various levels. A dominant absorption that is often attributed to O-H stretching vibration at 3361.74 of O-H in carboxylic acid which is accompanied with the bands at 2923.88 cm^{-1} corresponds to H stretching in carboxylic group. Exopolymers produced by marine bacteria generally contain 20–50% of the polysaccharide as uronic acid (Kennedy and Sutherland, 1987).

The band at 1740.04 cm^{-1} approves the stretching vibration of C = O carbonyl group of an aldehyde or ketone. Quite a spectral peak was obtained at 1628.99 cm^{-1} indicated amide NH_2 bending vibration or C = O, C = N stretching vibration of $RCONH_2$. The peak at 1370.45 cm^{-1} identifies the vibration stretching of alkyl hydrogen (CH_2-CH_3) in aliphatic alkyl group (R-CH_2-CH_3). The sharp band was observed at 1222.62 cm^{-1} strongly suggesting the stretching vibration of O-H group in a phenol. The peak at 1055.33 cm^{-1} is assigned

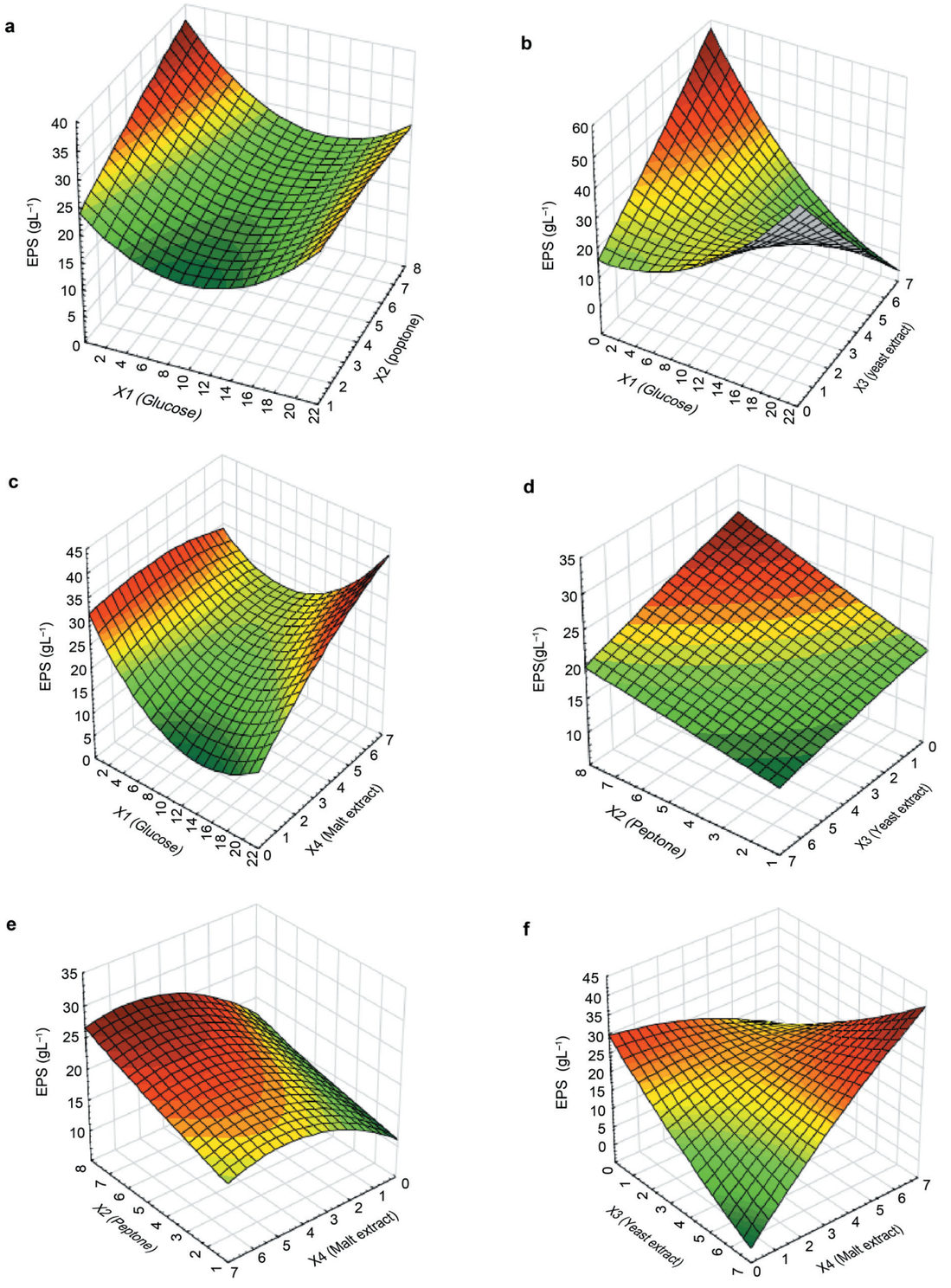

Fig. 4. Response surface plots of interaction between process variables in the EPSs production by *B. subtilis* SH1.

to stretching vibration of (C-O, alcohol, ester, ether and phenol) groups. It was reported that the EPS extracted from marine bacteria showed absorption indicated alkenes, ketones, isocyanate and isothiocyanate groups, alcohols, ethers, esters carboxylic acids and phenols groups (Orsod *et al.*, 2012).

UV spectrum analysis of EPSs (Fig. 7) indicated that there was a significant amount of absorption in

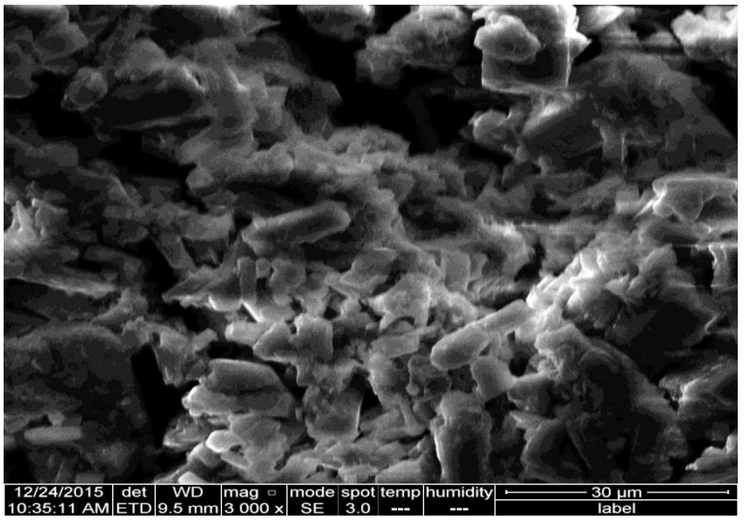

Fig. 5. Electron micrograph showing the surface of *B. subtilis* SH1 EPSs at 3000 X.

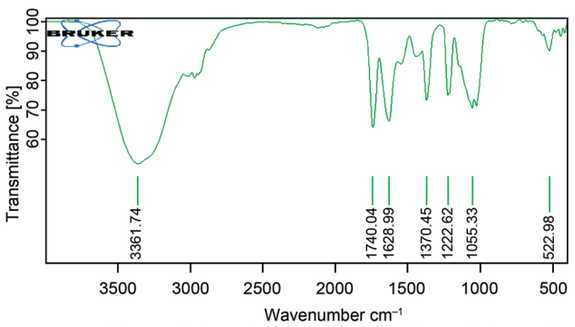

Fig. 6. FTIR spectra of the EPSs produced by *B. subtilis* SH1.

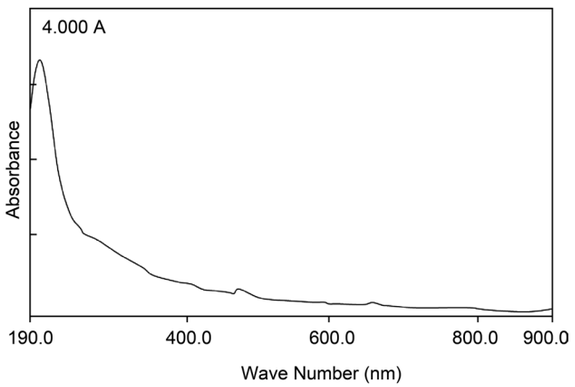

Fig. 7. UV analysis of the EPSs produced by *B. subtilis* SH1.

the UV area and revealed that the maximum absorption was at 190 nm. Trabelsi *et al.* (2009) stated that, the 190 ~ 230 nm wavelength area often results from n-σ* and/or π-π* transitions, which are found in many functional groups such as carboxyl, carbonyl, and ester.

The HPLC chromatogram of EPS in Fig. 8 showed the appearance of 2 major peaks, the first peak was at retention time 3.08 min with area 5.83% and the second peak was at retention time 9.65 min with area 91.23%. Based on the chromatogram, it was indicated that the EPS is a heteroploysaccharide contains maltose and rhamnose.

Different medical applications of the EPS produced by *B. subtilis* SH1. There are four types of activities detected and estimated for EPSs to be used as medical applications; antibacterial, antitumor, antiviral, and antioxidant activities.

Antibacterial activity. Different concentrations of the extracted EPS (10–200 mg/ml) were screened for antibacterial activity against different types of Gram-positive bacteria (*S. aureus*, *S. faecalis*) and Gram-negative bacteria (*E. coli*, *A. hydrophila*, *P. aeruginosa, V. damsela*). Data in Table IV showed that EPS had different range of antibacterial activity against the tested bacteria. The highest antibacterial activity (7.8, 9 and 10.4 AU/ml) was recorded against *S. faecalis* at 50, 100 and 200 mg/ml respectively while no inhibitory activity was detected against all the tested bacteria at lower concentrations (10 and 25 mg/ml). Antibacterial

Table IV
Antibacterial activity of the EPS from *B. subtilis* SH1 expressed as absolute unit (AU).

Indicator bacteria	AU/concentration (mg/ml)				
	10	25	50	100	200
A. hydrophila	ND	ND	3.6	5.4	5.4
P. aeroginosa	ND	ND	2.8	4.0	4.6
S. aureus	ND	ND	ND	ND	ND
V. damsela	ND	ND	ND	ND	ND
E. coli	ND	ND	ND	ND	ND
S. faecalis	ND	ND	7.8	9.0	10.4

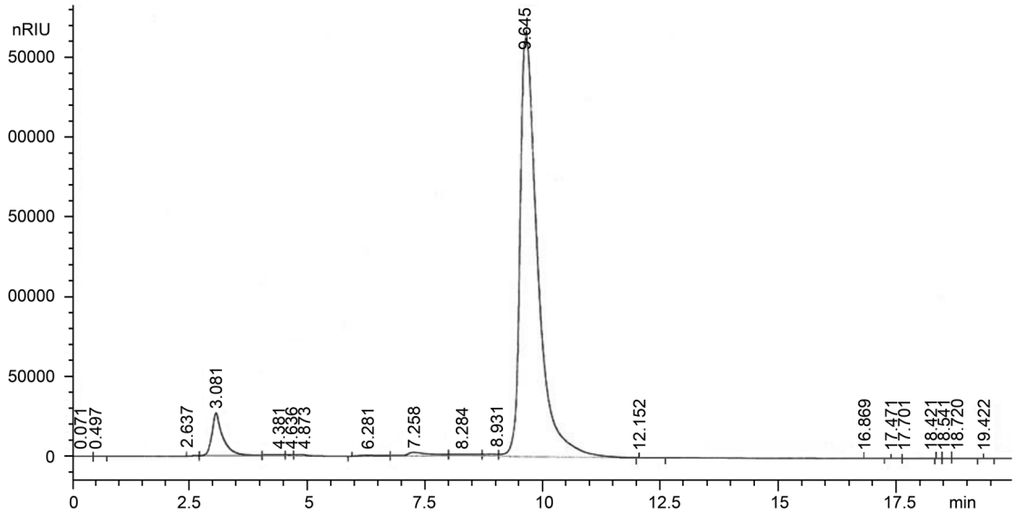

Fig. 8. Sugar analysis of EPS produced by *B. subtilis* SH1 using HPLC.

activity of EPS was proven in previous studies where Anju *et al.* (2010) isolated EPS from a marine bacterium with antibacterial activity against some fish pathogens. Also, Shankar *et al.* (2010) isolated the EPS from four biofilm bacteria and the isolated EPS exhibited antimicrobial activity against different pathogens. In addition, Orsod *et al.* (2012) reported that the extracted EPS from the marine bacteria which associated with Asian sea bass has potential antimicrobial activities against different pathogens.

Antitumor activity. The inhibitory effect of different concentrations (1.56, 3.13, 6.25, 12.5, 25 and 50 µg/ml) of EPS was tested on three different cell lines included MCF-7, HCT-116 and HepG2. Results showed that the produced EPS exhibited various degree of antitumor effect toward the tested cell lines and increasing concentrations of EPS resulted in increased rates of tumor inhibition. Results in Fig. 9A showed that, the cell viability of HCT-116 was 34.53–9 5.64% which exhibited inhibition % of 65.47–4.36% with IC_{50} = 35.8 µg which is consistent with the finding of You *et al.* (2011), where the obtained polysaccharide revealed a marked inhibition of proliferation of HCT 116. Regarding the antitumor effect of EPS against MCF-7, results in Fig. 9B showed that the

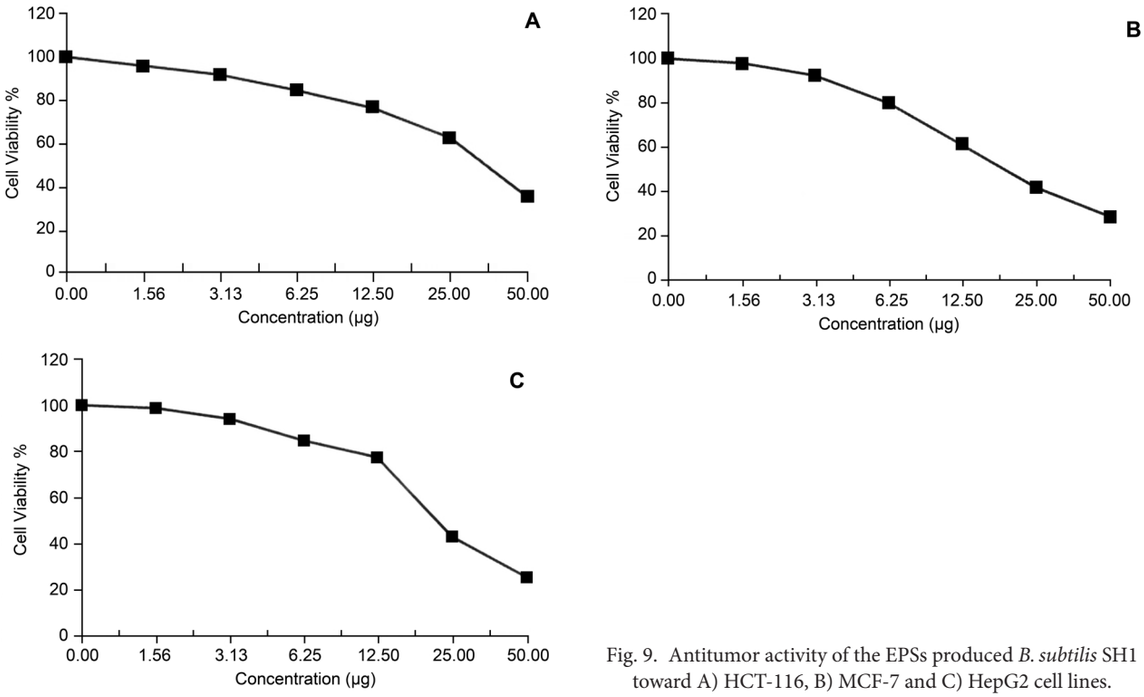

Fig. 9. Antitumor activity of the EPSs produced *B. subtilis* SH1 toward A) HCT-116, B) MCF-7 and C) HepG2 cell lines.

cell viability was 28.12 to 97.84% with inhibition range (71.88–2.16%) and exhibited $IC_{50} = 19.4$ µg

Fig. 9C shows that the viability % of the HepG2 cells ranged from 24.63 to 98.56% upon using EPS with different concentrations (0.39–50 µg) which means that it recorded inhibition range of (1.44–75.37%) and exhibited the highest antitumor effect compared to MCF-7 and HCT-116 cell lines with $IC_{50} = 22.3$ µg. The present work agree with that reported by You *et al.* (2011), who studied antitumor activity of different types of EPS toward 7 cell lines and reported that EPS exhibited the highest antitumor activity toward HepG2 (49.93–61.82%). The same result was reported by Dahech *et al.* (2012). It has been reported that the difference in antitumor effects of polysaccharides depends on their molecular weight, chemical composition, structure of the polymeric backbone, and degree of branching (You *et al.*, 2011).

Antiviral activity. Antiviral activity of EPS produced by *B. subtilis* SH1 was proven in different studies (Ahmed *et al.*, 2010). Polysaccharides are thought to inhibit the very early step of viral replication, *i.e.*, virus attachment to the cell surface (de Godoi, 2014). In the present study, EPS exhibited weak antiviral activity at 500 µg/ml against HSV-1, suggesting that this polysaccharide blocked a step in virus replication subsequent to virus attachment and entry. This result is in good agreement with the finding that increased internalization of heparin occurs during HSV-1 entry into cells to suggest that viral particles and the polysaccharide are co-internalized in endosomes. The polymer may inhibit a step in virus replication occurring between virus release and the synthesis of late proteins.

Antioxidant activity. The antioxidant capacity of EPS was determined by DPPH (2, 2-Diphenyl-2-picryl-hydrazyl) and revealed that, the sample has a significant antioxidant activity compared with ascorbic acid as control (Fig. 10). The different concentrations of the EPS sample (128, 64, 32, 16, 8, 4, 2, and 1 µg/ml) showed different percentages of inhibition (89.89, 88.86, 87.95, 81.03, 69.35, 38.49, 23.68, and 14.92%), respectively. These results revealed that, the antioxidant capacity was increased with increasing the concentration of the sample. These results agree with that recorded by Abdel-Fattah *et al.* (2012), who stated that the antioxidant activity of levan and their derivatives (SL1 and SL2) produced by *B. subtilis* exhibited a strong free radical scavenging activity with DPPH. You *et al.* (2011) also reported the antioxidant activity of different types of polysaccharides.

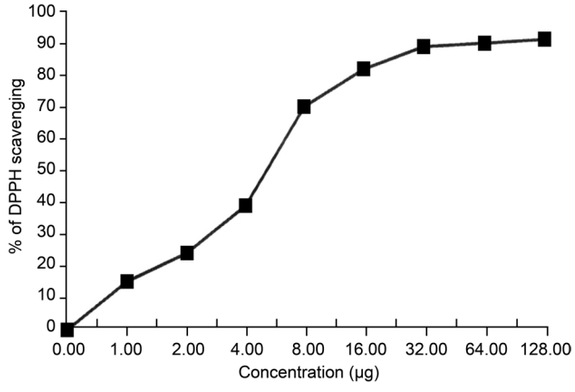

Fig. 10. Antioxidant activity of the EPSs produced by *B. subtilis* SH1.

Conclusions

The present study is spotlight on the importance of EPS from *B. subtilis* SH1 and the data confirmed the following points:

1. The YMG medium was the most suitable for higher production of *B. subtilis* SH1 EPSs.
2. The production of EPS increased gradually from the first day to the fourth day recording the highest productivity and the highest productivity of EPS was also favorable at the alkaline level.
3. The maximum productivity of EPS was achieved using RSM design at conditions of low concentrations of glucose and peptone but with high concentrations of yeast extract and malt extract.
4. The structural characterization was done by FTIR spectroscopy which exhibited bands at various levels. HPLC revealed the presence of maltose and rhamnose as major component in EPS.
5. EPS produced by *B. subtilis* SH1 showed valuable activities for medical purposes such as antibacterial, antitumor, antiviral, and antioxidant.

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ORIGINAL PAPER

Characterisation of Antimicrobial Properties of Extracts of Selected Medicinal Plants

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Abstract

The scope of the experiments included analysis of the antimicrobial activity of ethanolic, methanolic and aqueous extracts against bacterial and fungal cultures and determination of the minimum inhibitory concentration of plant extracts tested microbial growth. Analysis of the antifungal and antibacterial activity was carried out by the disc diffusion method using paper discs. In the experiment 11 species of microorganisms – 8 bacterial and 3 fungal strains were used. The highest antimicrobial activity against the tested strains was demonstrated by black elder (*Sambucus nigra* L.), black locust (*Robinia pseudoacacia* L.) and lingonberry (*Vaccinium vitis-idaea* L.) extracts. The study showed the diverse morphological activity of specific parts of elderberry and quince, which is the effect of different polyphenolic profile of these plants. The yeast *Saccharomyces cerevisiae*, *Escherichia coli*, *Pseudomonas putida* and *Bacillus subtilis* showed the highest sensitivity to the effect of extracts of the analysed plants. As a positive control three antibiotics – amphotericin B, vancomycin and amoxicillin with clavulanic acid were used.

Key words: *Robinia pseudoacacia* L., *Sambucus nigra* L., *Vaccinium vitis-idaea* L., antimicrobial activity, medicinal plants

Introduction

Medicinal plants contain a number of valuable substances, which can support the prevention and treatment of various diseases. Due to differences in the chemical composition of the mixture, biologically active substances exhibit the activity of a different type than acting separately, which is the result of synergism or antagonism of their various components.

The main components responsible for the antimicrobial properties of plants are polyphenolic compounds. They exhibit anti-inflammatory activity *in vitro* and *in vivo* and their mechanism of action is the inhibition of enzymes (phospholipase oxygenase) *i.e.* by binding with hydrosulfide groups and inactivation of bacterial proteins (Kim *et al.*, 1995; Cowan, 1999).

Antimicrobial activity is attributed to flavonols, quinones and flavonoids. These substances exhibit lipophilic properties and cause the destruction of the cell wall and cytoplasmic membrane of microorganisms. Furthermore, they cause inhibition of nucleic acid synthesis, structural and enzymatic proteins as well as saccharides. It has been shown that the antimicrobial activity of flavonoids may be dependent on their structure. It is believed that unsubstituted flavones are characterized by the highest antifungal activity, and flavanones by lower. The introduction to these compounds of hydroxyl or methyl groups reduces their antifungal properties (Małolepsza and Urbanek, 2000).

Anthocyanins form complexes with metals, participating in this way in shaping the colour of flowers (Mitek and Gasik, 2009; Quina *et al.*, 2009). The bioavailability and biological activity of these compounds is closely related to their chemical structure, which affects a large variety of them. Studies have shown that plant extracts rich in these substances have antibacterial and antitumor activity (Middleton *et al.*, 2000; Olejnik *et al.*, 2009).

Secondary metabolites of plants responsible for their antimicrobial properties can also include alkaloids (berberine) which damage the DNA of microbial cells, leading to their death (Omulokoli *et al.*, 1997).

Antimicrobial activity of the protein rather consists of the formation of ion channels in the cell membrane of the microorganism, increasing its permeability. The mechanism of action may also involve blocking the

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metabolism of the essential compounds for the bacteria and the inhibition of adhesion microorganism to the surface of the plant cell (Cowan, 1999).

Tannins protect a plant against the effects of microorganisms by the formation of complexes with proteins, while terpenes interfere with the incorporation of the lipophilic compounds in the bacterial cell membrane (Mendoza *et al.*, 1997).

On the basis of phytochemical analysis the presence of many biologically active chemical compounds in flowers, leaves, fruits and rhizomes of medicinal plants was discovered. As a natural source of valuable substances for health and readily available ones, they are an object of interest of pharmacological studies, showing tremendous therapeutic potential.

The aim of the study was to determine the antimicrobial effects of extracts of selected medicinal plants and their minimum dose that inhibits growth of 11 species of microorganisms, both bacterial and fungal.

Experimental

Materials and Methods

Plant material and preparation of extracts. In this study ethanolic, methanolic and aqueous extracts made of fruits of dogwood (*Cornus mas* L.), black mulberry (*Morus nigra* L.), lingonberry (*Vaccinium vitis-idaea* L.), flowers of hawthorn (*Crataegus oxyacantha* L.), black locust (*Robinia pseudoacacia* L.), fruits and leaves of quince (*Cydonia oblonga* Mill.) and leaves and flowers of black elder (*S. nigra* L.) were used. Analysed medicinal plants were collected from organic farming in Lesser Poland Voivodeship. Suitable for analysis parts of plants were harvested and frozen at –80°C, in order to inhibit transformation of the antioxidant activity of the compounds. Frozen research material (10.0 g) was weighed into conical flasks with ground glass joint. Fruits of quince and dogwood were divided after thawing into smaller parts. To the analysed material (in a flask) 90 ml of a suitable solvent (ethanol 80% vol., methanol 80% vol. or hot water) was poured over and extracted with a magnetic stirrer (6 h, room temp.). After extraction, the solutions were filtered, adjusted to 100 ml with a suitable solvent for the determination and stored at –20°C. Extraction of each material was conducted with 3 replications.

Microorganisms. For the tests 11 species of microorganisms (8 bacteria and 3 fungi) from the Pure Culture Collection of the Department of Fermentation Technology and Technical Microbiology, University of Agriculture in Krakow were used. Bacterial cultures: *Bacillus subtilis* subsp. *subtilis* DSM 10, *Bifidobacterium* sp. DSM 20104, *Clostridium* sp. DSM 756, *Escherichia coli* DSM 4261, *Micrococcus luteus* DSM 20030, *Proteus myxofaciens* DSM 4482, *Pseudomonas putida* DSM 291, *Serratia marcescens* DSM 1636. Fungal cultures: *Aspergillus niger* CBS 10930, *Penicillium chrysogenum* DSM 844, *Saccharomyces cerevisiae* DSM 1333.

Microbiological media. Aerobic bacterial strains were cultivated at 32°C for 24 hours and tested on tryptic soy agar (Merck, Germany), and fungal cultures at 28°C for 48 hours on Sabouraud agar (Merck, Germany). Anaerobic *Bifidobacterium* sp. was cultivated at 37°C for 18 hours and grown on the Bifidobacterium-Medium (g/l water: casein peptone, 10; yeast extract, 5; beef extract, 5; soybean hydrolyzate, 5; glucose, 10; K_2HPO_4, 2; $MgSO_4 \cdot 7H_2O$, 0.20; $MnSO_4 \cdot H_2O$, 0.05; Tween 80, 1; NaCl, 5; L-cysteine, 0.50; salt solution, 40; Resazurin (25 mg/100 ml), 4 and *Clostridium* sp. on Pyx-agar medium (g/l water: tryptone, 5; peptone, 5; yeast extract, 10; glucose, 5; resazurin (25 mg/100 ml), 1; salt solution, 40; L-cysteine, 0.50). The composition of the salt solution in g/l of water: $CaCl_2 \cdot 2H_2O$, 0.25; $MgSO_4 \cdot 7H_2O$, 0.50; K_2HPO_4, 1; KH_2PO_4, 1; $NaHCO_3$, 10; NaCl, 2).

Determination of antimicrobial activity of extracts of analysed medicinal plants. The analysis of the antifungal and antibacterial activity was carried out by using a diffusion paper-disc assay. 24-hour age bacterial (10^9 CFU/ml) or fungal (10^8 CFU/ml) culture was streaked into appropriate media, next the paper discs with a diameter of 14 mm were placed and soaked with 4 mg/ml of the analysed extract. The 80% vol. solutions of ethanol and methanol were the negative control, while the positive were discs with vancomycin (30 mg, Bioanalyse, Turkey) or amoxicillin with clavulanic acid (30 mg, Biolab Zrt., Hungary) for bacteria and amphotericin B (10 mg, Sigma-Aldrich, Germany) for fungi. Plates were incubated for 18 h at 37°C (bacteria) and 48 h at 28°C (fungi). Anaerobic microorganisms were plated on Petri dishes by submerged method. Subsequent procedure was analogous to the above. The strains were incubated under anaerobic conditions for 48 h at 37°C. The antimicrobial activity was defined as the diameter of growth inhibition zone around the disks of blotting paper soaked with plant extracts.

Determination of the minimum inhibitory concentration of the plant extracts (MIC). MIC was determined by the classical method in the solid growth medium. 24-hour old bacterial cultures (10^9 CFU/ml) cultivated at 28°C and 48-hour old fungal cells (10^8 CFU/ml) at 37°C and were plated into growth media on which paper disks with a diameter of 14 mm, soaked with 1, 2, 3 or 4 mg/ml respectively of the appropriate extract were placed. Negative control were 80% vol. ethanol and methanol solutions. The plates were incubated for 24 h at 28°C (bacteria) and for 48 h at 37°C (fungi). Anaerobic microorganisms were plated

on Petri dishes by submerged method. Subsequent procedure was analogous to the above. The strains were incubated under anaerobic conditions for 48 h at 37°C. MIC was defined as the lowest extract concentration that inhibited the growth of microorganisms on a solid growth medium.

Statistical analysis. Variance analysis was used for the obtained results analysis (Statistica program). Comparisons between groups were performed using the post-hoc test of Scheffe. To performed similarity analysis was used dendritic method. All assays were performed in 9 replications.

Results

The analysed extracts were characterized by diverse antifungal and antibacterial activity. The methanol and ethanol extracts proved to be most effective in inhibiting the growth of tested microbial cultures. Extracts from lingonberry and black locust fruits as well as black elder flowers showed the highest antimicrobial activity with respect to the analysed fungal and bacterial cultures. Other plant species had significantly lower antimicrobial properties.

Antimicrobial activity of analysed plants extracts are presented in Table I. Antibiotic effect depended primarily on the type of solvent used for extraction. After eliminating its influence on the culture of microorganism in all cases the most significant zone of growth inhibition of microorganisms was found in case of ethanol and methanol extracts. Extracts of black elder flowers as well black locust and lingonberry fruits most effectively inhibited the growth of tested microorganisms. Contrary to it the activity of black mulberry fruits extracts was the weakest. It inhibited only 8 cultures.

S. cerevisiae yeast were the most sensitive to the effects of extracts from black elder, hawthorn and common quince. To a much lesser extent, these extracts inhibited the growth of bacterial cultures. The only exception were *P. myxofaciens* and *P. putida* showing high sensitivity to the extract from the fruit of *S. nigra*.

Table II shows the antimicrobial action of antibiotics – vancomycin (30 mg) and amoxicillin (20 mg) for bacteria and amphotericin B (10 mg) for fungi used as positive control. These substances blocked effectively cell proliferation of used microorganisms except amoxicillin, which does not affect the *B. subtilis* bacteria.

Ethanol, methanol and water extracts derived from black elder characterized by a relatively growth broad antimicrobial activity (Table III). The extracts of *S. nigra* flowers were slightly worse inhibitors of microbial growth than those obtained from the fruits.

Methanol extracts were characterized by antibiotic activity only against *S. cerevisiae* yeast and bacteria of *E. coli*, *P. myxofaciens*, *M. luteus*, *P. putida* and *Bifidobacterium* sp. In all cases, the minimum inhibitory dose was 2 mg/ml.

Extracts of hawthorn flower were distinguished by slightly weaker antimicrobial activity (Table IV) but strongly inhibited the development of *S. cerevisiae* and *E. coli* (MIC 2 mg/ml). These extracts impacted the growth of other analysed microorganisms. Aqueous extracts of hawthorn flowers demonstrated efficacy against *S. cerevisiae* yeast (MIC 2 mg/ml).

The highest antimicrobial activity was observed in the case of extracts from black locust flowers (Table V). Like the other analysed plants, they did not affect the growth of *A. niger* and *P. chrysogenum* as well as *P. myxofaciens* and *S. marcescens*. The ethanol extracts of black locust effectively inhibited the growth of other microorganisms used in the experiments. In the case of most microorganisms, their growth was inhibited by concentration of 2 mg/ml.

Extract from lingonberry fruit showed relatively high antimicrobial activity (Table VI), however in some cases such as *E. coli*, *M. luteus*, *P. putida*, *Bifidobacterium* sp. and *Clostridium* sp. higher MIC compared to the other extracts discussed above was detected. Noteworthy, that the growth of *A. niger* was stopped by ethanol and methanol lingonberry extracts. Likewise extracts of elderberry fruit, lingonberry extracts inhibited most effectively development of *P. myxofaciens* bacteria (MIC 2 mg/ml). Aqueous extracts showed antimicrobial activity in higher concentration (> 4 mg/ml).

Elderberry, black locust and black mulberry were characterized by the highest antimicrobial activity against tested bacteria and fungi. The study also showed various activity of different morphological parts of elderberry and quince which might be the effect of different polyphenols profile of these plants. The *S. cerevisiae* yeast and bacterial cultures of *E. coli*, *P. putida* and *B. subtilis* were characterized by the highest sensitivity on the tested plant extracts. As a positive control 3 antibiotics were used – amphotericin B, vancomycin and amoxicillin with clavulanic acid.

It was found that amphotericin B inhibited the growth of all tested fungal cultures (Table II). In the case of *S. cerevisiae* there was observed approximately 2-fold higher inhibition zone compared to *A. niger* and *P. chrysogenum*. Studies on the amphotericin B mechanism of action on yeast strains revealed that this antibiotic induces the stimulation of cells, permeabilization of cell membranes and can lead to death of microorganism.

Statistical analysis. Based on the performed similarity analysis by dendritic method, the sensitivity of tested microorganisms was classified regard to ethanol, methanol and water plants extracts (Fig. 1, 2, 3). Separated groups allow effectively present mutual similarities and significant differences in action of plant extracts against

Table I
Antimicrobial activity of ethanol (E), methanol (M) and water (W) extracts of analyzed plants against selected strains (inhibition zones in mm)

Plant material[y]		*Saccharomyces cerevisiae*	*Penicillium chrysogenum*	*Aspergillus niger*	*Escherichia coli*	*Serratia marcescens*	*Proteus myxofaciens*	*Micrococcus luteus*	*Pseudomonas putida*	*Bacillus subtilis*	*Bifidobacterium* sp.	*Clostridium* sp.	Sig.[z]
Black elder (flower)	E	0.78 ^{a}	0.17 ^{b}	0	0	0	0.17 ^{b}	0.17 ^{b}	0.11 ^{b}	0	0	0	**
	M	0.11 ^{b}	0	0	0.17 ^{b}	0	0.5 ^{b}	0.22 ^{a}	0.22 ^{a}	0	0	0	**
	W	0.94 ^{ab}	0	0	1.44 ^{a}	0.94 ^{ab}	1.44 ^{a}	1.33 ^{a}	1.22 ^{a}	1.17 ^{a}	0.22 ^{c}	0.11 ^{c}	*
Hawthorn (flower)	E	1.5 ^{a}	0	1.33 ^{a}	1.22 ^{a}	0	0.11 ^{b}	0	0.17 ^{b}	0	0	0	**
	M	0.17 ^{b}	0	0	0	0.28 ^{a}	0.28 ^{a}	0	0	0.5 ^{a}	0	0	**
	W	0.67 ^{bc}	0	0	1.5 ^{a}	1.5 ^{a}	1.39 ^{a}	0.89 ^{ab}	1.39 ^{a}	0.89 ^{ab}	0	0.11 ^{c}	*
Locust (flower)	E	0	0	0	0	0	0	0	0	0	0.06 ^{b}	0.11 ^{a}	**
	M	0	0	0	0	0	0.33 ^{a}	0	0.17 ^{a}	0	0.06 ^{b}	0	**
	W	1 ^{ab}	0	0	1.5 ^{a}	1.33 ^{a}	1.28 ^{a}	0.89 ^{ab}	1 ^{ab}	0.89 ^{ab}	0.11 ^{c}	0.44 ^{c}	*
Cowberry (fruit)	E	0	0	0	1.11 ^{a}	0.11 ^{b}	0.28 ^{b}	0	0	0.5 ^{a}	0	0	**
	M	0.39 ^{a}	0	0	0	0	0.5 ^{a}	0	0	0.28 ^{a}	0.06 ^{b}	0	*
	W	0	0	0	1.5 ^{a}	0.22 ^{b}	1.44 ^{a}	0.33 ^{b}	0.67 ^{b}	1.17 ^{a}	0	0.33 ^{b}	**
Mulberry (fruit)	E	0.89 ^{a}	0	0	0.28 ^{b}	0	1 ^{a}	0.78 ^{a}	0	0	0	0	**
	M	0	0	0	0	0	0.5 ^{a}	0.33 ^{a}	0.28 ^{a}	0.33 ^{a}	0	0.06 ^{b}	**
	W	0.67 ^{bc}	0	0	1.67 ^{a}	0	1 ^{ab}	1.5 ^{a}	1.17 ^{a}	1.67 ^{a}	0.11 ^{c}	0.44 ^{bc}	*
Quince common (fruit)	E	0	0	0	0.88 ^{a}	0	0.06 ^{b}	0	0.28 ^{a}	0	0	0	**
	M	0.17 ^{b}	0	0	0	0	0.39 ^{a}	0	0	0.56 ^{a}	0	0	**
	W	1.5 ^{a}	0	0	0.94 ^{ab}	0.56 ^{bc}	1.17 ^{a}	0.5 ^{bc}	1.22 ^{a}	1.39 ^{a}	0.22 ^{c}	0.56 ^{bc}	*
Dogwood (fruit)	E	0.72 ^{a}	0	0	0.67 ^{a}	0	0	0.06 ^{b}	0	0	0	0	**
	M	0.17 ^{a}	0	0	0	0	0.22 ^{a}	0.11 ^{b}	0	0.39 ^{a}	0	0	**
	W	0.67 ^{c}	0	0	1 ^{ab}	0.67 ^{bc}	1.28 ^{a}	1.44 ^{a}	1.44 ^{a}	1.33 ^{a}	0.22 ^{c}	0.11 ^{c}	*
Black elder (fruit)	E	1.22 ^{a}	0	1.83 ^{a}	0.28 ^{b}	0	1.11 ^{a}	1.17 ^{a}	0	0	0	0	**
	M	0.17 ^{b}	0	0	0	0	0.22 b	0.5 a	0	0.44 a	0	0	**
	W	0	0.44 ^{bc}	0	1.28 ^{a}	0.94 ^{ab}	1.28 ^{ab}	1.5 ^{a}	0.78 ^{ab}	0.94 ^{ab}	0.56 ^{bc}	0.22 ^{c}	*
Quince common (leaves)	E	0.06 ^{b}	0	0.28 ^{b}	0	0.83 ^{a}	0.5 ^{a}	0	0	0	0	0	**
	M	0	0	0	0	0	0.5 ^{a}	0	0.39 ^{b}	0	0	0	**
	W	1.33 ^{a}	0.22 ^{c}	0	1.17 ^{ab}	1.11 ^{ab}	1.44 ^{a}	1 ^{ab}	1.22 ^{a}	1.22 ^{a}	0.56 ^{bc}	0	*

[Y] According to the Scheffe test. means within columns followed by the same letter are not significantly differen;
[Z] Sig: Significance; * and ** display the significance at 5 % and 1 % respectively.

Table II
Antimicrobial activity of selected antibiotics against analyzed microbial strains (inhibition zones in mm; mean of three replicates)

Antibiotic substance	*Saccharomyces cerevisiae*	*Penicillium chrysogenum*	*Aspergillus niger*	*Escherichia coli*	*Serratia marcescens*	*Proteus myxofaciens*	*Micrococcus luteus*	*Pseudomonas putida*	*Bacillus subtilis*	*Bifidobacterium* sp.	*Clostridium* sp.
Amoxicillin (30 µg)	–	–	–	0.22	0.22	0.88	0.33	0.22	0	1.17	1.28
Vancomycin (20 µg)	–	–	–	1.89	1.22	0.83	0.67	0.67	4.44	9.56	8.78
Amfotericin B (100 µg)	7.61	4.11	3.56	–	–	–	–	–	–	–	–

Table III
Antimicrobial activity of different concentrations of black elder flower ethanol (E), methanol (M) and water (W) extracts against the analyzed microbial strains (inhibition zones in mm; mean of three replicates)

Plant material [mg/ml]			*Saccharomyces cerevisiae*	*Penicillium chrysogenum*	*Aspergillus niger*	*Escherichia coli*	*Serratia marcescens*	*Proteus myxofaciens*	*Micrococcus luteus*	*Pseudomonas putida*	*Bacillus subtilis*	*Bifidobacterium* sp.	*Clostridium* sp.
Black eder (flower)	E	2.0	0.50	–	–	0.17	–	–	0.17	–	–	0.11	–
		3.0	0.83	–	–	0.50	–	–	–	–	–	–	–
		4.0	1.17	–	–	–	–	–	0.17	–	–	–	–
	M	2.0	–	–	–	–	–	0.50	0.17	0.33	–	0.17	0.11
		3.0	–	–	–	–	–	–	–	–	–	0.06	–
		4.0	–	–	–	–	–	0.50	–	0.33	–	0.22	–
	W	2.0	0.50	–	–	–	–	–	–	–	0.50	–	–
		3.0	1.00	–	–	–	–	–	–	–	1.00	–	–
		4.0	1.50	–	–	1.00	1.00	–	–	1.00	1.50	–	–

Table IV
Antimicrobial activity of different concentrations of hawthorn flower ethanol (E), methanol (M) and water (W) extracts against the analyzed microbial strains (inhibition zones in mm; mean of three replicates)

Plant material [mg/ml]			*Saccharomyces cerevisiae*	*Penicillium chrysogenum*	*Aspergillus niger*	*Escherichia coli*	*Serratia marcescens*	*Proteus myxofaciens*	*Micrococcus luteus*	*Pseudomonas putida*	*Bacillus subtilis*	*Bifidobacterium* sp.	*Clostridium* sp.
Hawthorn (flower)	E	2.0	0.83	–	–	0.17	0.17	0.17	0.17	–	–	–	–
		3.0	1.17	–	–	0.50	–	–	–	–	–	0.17	–
		4.0	1.50	–	–	–	0.17	0.17	0.17	–	–	–	–
	M	2.0	0.33	–	–	0.17	–	–	–	0.17	–	0.33	–
		3.0	–	–	–	–	–	–	–	–	–	–	–
		4.0	0.33	–	–	–	–	–	–	0.17	–	0.22	–
	W	2.0	0.50	–	–	–	–	–	–	–	–	–	–
		3.0	1.00	–	–	–	–	–	–	–	–	–	–
		4.0	1.50	–	–	1.00	–	–	–	1.00	–	–	–

Table V
Antimicrobial activity of different concentrations of black locust flower ethanol (E), methanol (M) and water (W) extracts against the analyzed microbial strains (inhibition zones in mm; mean of three replicates)

Plant material [mg/ml]			*Saccharomyces cerevisiae*	*Penicillium chrysogenum*	*Aspergillus niger*	*Escherichia coli*	*Serratia marcescens*	*Proteus myxofaciens*	*Micrococcus luteus*	*Pseudomonas putida*	*Bacillus subtilis*	*Bifidobacterium* sp.	*Clostridium* sp.
Black locust (flower)	E	2.0	0.50	–	–	0.50	–	–	–	–	–	0.22	–
		3.0	0.83	–	–	1.00	–	–	–	0.50	–	–	–
		4.0	1.00	–	–	0.17	–	–	–	0.17	–	0.11	–
	M	2.0	0.83	–	–	–	–	–	0.17	0.50	–	0.22	0.22
		3.0	0.67	–	–	–	–	–	–	–	0.33	–	–
		4.0	0.83	–	–	–	–	–	–	0.50	0.17	0.11	–
	W	2.0	–	–	–	–	–	–	–	–	–	–	0.33
		3.0	–	–	–	1.00	–	–	1.00	–	–	–	0.11
		4.0	–	–	–	1.00	1.00	–	1.00	–	1.50	–	0.44

Table VI
Antimicrobial activity of different concentration of cowberry fruit ethanol (E), methanol (M) and water (W) extracts against the analyzed microbial strains (inhibition zones in mm; mean of three replicates)

Plant material [mg/ml]			*Saccharomyces cerevisiae*	*Penicillium chrysogenum*	*Aspergillus niger*	*Escherichia coli*	*Serratia marcescens*	*Proteus myxofaciens*	*Micrococcus luteus*	*Pseudomonas putida*	*Bacillus subtilis*	*Bifidobacterium* sp.	*Clostridium* sp.
Cowberry (fruit)	E	2.0	0.83	–	0.50	–	–	0.33	–	–	–	–	0.11
		3.0	0.83	–	–	–	0.50	0.33	–	–	0.33	0.17	–
		4.0	0.67	–	0.17	–	0.50	0.67	–	–	–	0.06	–
	M	2.0	0.17	–	0.17	0.17	–	–	0.17	–	–	–	–
		3.0	0.17	–	0.67	–	–	–	–	–	0.67	0.11	–
		4.0	0.33	–	0.83	–	–	–	–	–	0.33	0.11	–
	W	2.0	–	–	–	–	–	–	–	–	–	–	–
		3.0	–	–	–	–	–	–	–	–	–	–	–
		4.0	–	–	–	1.00	–	1.00	–	1.00	1.00	–	0.22

individual bacteria and fungi strains. The results indicate the diverse response of the tested microorganisms to ethanol, methanol and aqueous plant extracts. The highest similarity with respect to the relative sensitivity to the analysed ethanol extracts was shown by 4 groups of microorganisms (Fig. 1). The first included anaerobic microorganisms (*Bifidobacterium* sp., *Clostridium* sp.) and filamentous fungus *P. chrysogenum*. The second group was aerobic bacteria (*P. putida*, *B. subtilis*, *S. marcescens*, *P. myxofaciens*, *M. luteus*), which were characterized by low sensitivity to the analysed plant ethanol extracts. The third group – *S. cerevisiae* and *A. niger*. *E. coli* formed a separate group with a significant Euclidean distance from other microorganisms.

The results of the cluster analysis of methanol plant extracts is shown in Fig. 2. In this case, clusters were determined bacterial microorganisms. The first of these include *S. marcescens* and anaerobic *Clostridium* sp. and *Bifidobacterium* sp. bacteria. *S. cerevisiae* formed a separate group with a significant Euclidean distance from other microorganisms. Aqueous extracts (Fig. 3) were the most homogeneous group with respect to observe the highest similarity of analysed microorganisms. *P. chrysogenum* and *A. niger* strains, as well as anaerobic *Clostridium* sp. and *Bifidobacterium* sp. bacteria proved to be the least sensitive to action of water plant extracts. Other microorganisms have shown inhibition relative to the tested aqueous extracts.

Discussion

The experiments included the antimicrobial activity of ethanolic, methanolic and aqueous extracts against selected bacterial and fungal cultures as well as evaluation of minimum inhibitory concentration of plant extracts. Analysis of antifungal and antibacterial activity was carried out by disc diffusion using paper discs. In the experiment 11 species of microorganisms were used – 8 bacterial and 3 fungal strains.

Literature data reported that extracts of black elder flowers as well fruits inhibited Gram-positive (*Staphylococcus aureus*, *Bacillus cereus*) and Gram-negative bacteria (*Salmonella poona*, *Pseudomonas aeruginosa*). Zone of growth inhibition hesitate from 5 to 14 mm. Flavonons, flavonols dihydroflavonols present in many flowers may be responsible for their antimicrobial properties. Furthermore, they can contain lecithin, peptides and oligosaccharides which are inhibitors of transcription and metabolism of the bacterial cells (Hearst *et al.*, 2010).

Experiments of Oliveira *et al.* (2007) showed that methanol extracts of black elder did not inhibit the growth of *B. subtilis*, *P. aeruginosa*, *Aeromonas hydrophila* and *S. aureus*. However, during studies conducted

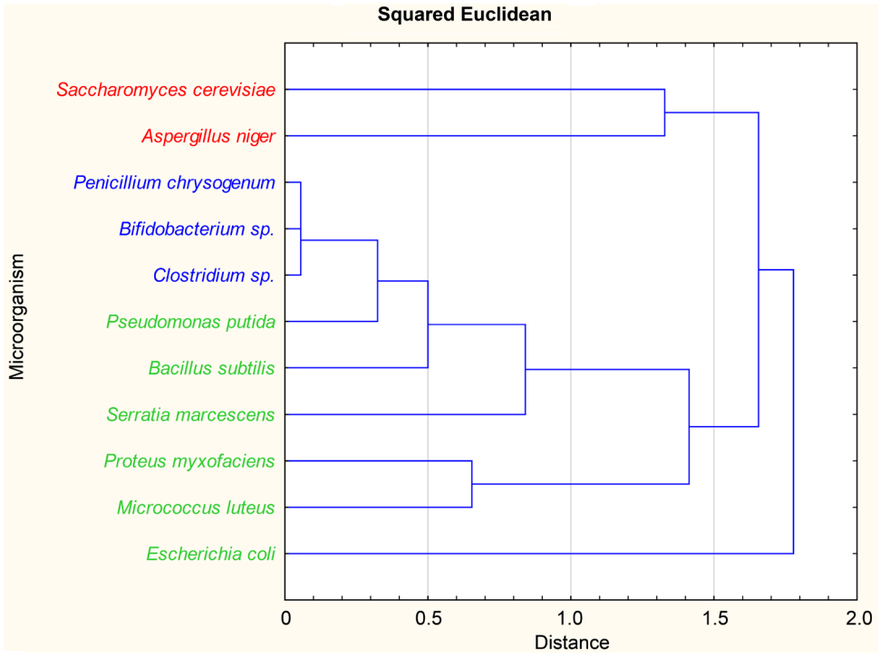

Fig. 1. Diagram of similarities of tested microorganisms with respect to ethanol plant extracts made by dendritic method.

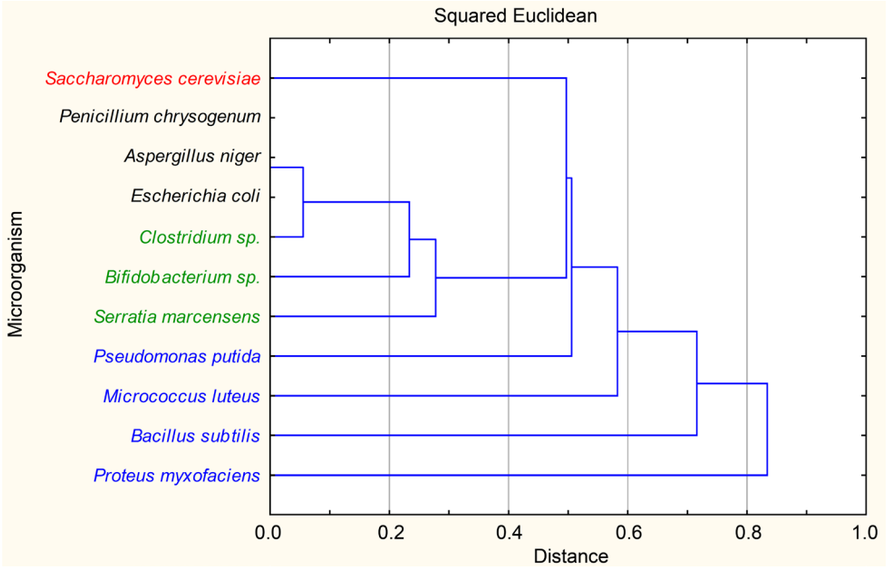

Fig. 2. Diagram of similarities of tested microorganisms with respect to methanol plant extracts made by dendritic method.

by the Bussmann *et al.* (2010) ethanol extract of *S. nigra* slightly inhibited the growth of *S. aureus*, while no inhibition was found in the case of *E. coli*.

The extracts obtained from different organs of the same plant exhibited various action against microorganisms, for example leaves of quince extracts were characterized by better antimicrobial activity than the fruit extracts.

Research conducted by Silva *et al.* (2005) indicates a different polyphenol composition of individual morphological parts of quince. In *Cydonia oblonga* leaves the presence of flavonol glycosides, phenolic acids and derivatives of quercetin were detected, while in fruits concentration of these components was significantly lower. Noteworthy is the fact that the composition of the various parts of the same plant is directly related to

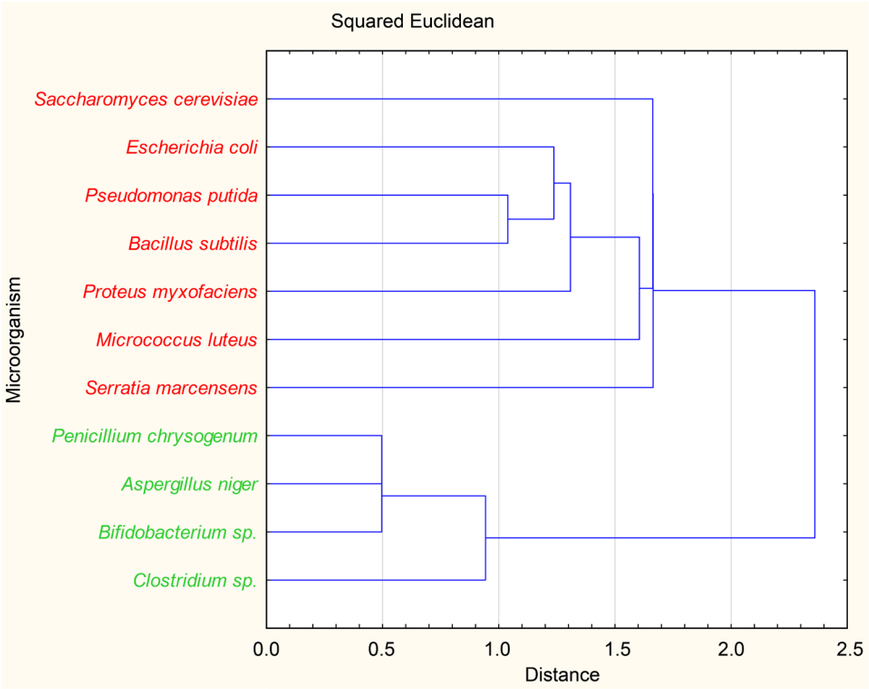

Fig. 3. Diagram of similarities of tested microorganisms with respect to water plant extracts made by dendritic method.

its exposure to environmental conditions and is associated with the degree of its maturity. The content of polyphenolic compounds changes during growth and ripening of quince, which may affect its antimicrobial properties (Wojdyło, 2011).

Aqueous extracts of black elder flowers, in contrast to the fruit, inhibited the growth of *S. cerevisiae* yeast, whereas the latter showed the action against anaerobic bacteria. There were no zones of inhibition around the disks soaked with extracts in the case of *A. niger* and *P. chrysogenum*. Ethanol extracts affected to a greatest extent the growth of *S. cerevisiae* yeasts and *E. coli* bacteria with MIC for these microorganisms being 2 mg/ml. The growth of anaerobic bacteria of the genus *Bifidobacterium* sp. and *Clostridium* sp. was inhibited strongly by aqueous extract of black elder fruit.

Because of all parts of the black elder contain sambunigrin, a toxic compound present mainly in the immature fruit and cyanogenic glycosides (prunasin), it is believed that the plant extracts may have antifungal activity (Charlebois *et al.*, 2010). It has been also shown that the antifungal PR 1 proteins which occur in black elder can bind to the protein channels in cell membranes, cause disturbances in the release of calcium ions, thereby have antifungal properties against plant pathogens such as *Uromyces fabae* and *Erysiphe graminins* (Selitrennikoff, 2001).

Hawthorn flowers, like fruits of elderberries contain phenolic acids (chlorogenic acid), quercetin-3-galactoside derivatives, catechins, and a high concentration of flavonoids. In experiments conducted by Proestos *et al.* (2008) was found that plant extracts of hawthorn have little impact against *S. aureus* and *Salmonella enteritidis* and do not inhibit the growth of *E. coli* O157: H7, *B. cereus* and *P. putida* (Aboaba *et al.*, 2008).

The evaluation of antibacterial activity of *Crataegus oxyacantha* showed that the tested bacterial strains of *E. coli*, *P. aeruginosa*, *S. aureus*, *Klebsiella pneumoniae* and *Proteus mirabilis* are sensitive to this plant extracts and the O-glycosides of flavonols were the main components responsible for its antimicrobial properties (Mercincak *et al.*, 2008).

In comparison to the previously discussed extracts, flowers of *Robinia pseudoacacia* showed a higher activity against *P. putida*. Aqueous extracts, in contrast to methanol and ethanol strongly inhibited the growth of *B. subtilis*.

In this study, the higher sensitivity of *E. coli*, *S. cerevisiae* and *P. myxofaciens* on aqueous extracts of this plant compared to methanol was found. Similar results were obtained by Zhang *et al.* (2008), they demonstrated the antibacterial effect of aqueous extracts of black locust against *Staphylococcus* and coli forms as well as antifungal properties against *Plasmopara viticola*.

Ethanol extracts of quince leaves did not act on filamentous fungi, while they influenced stronger than fruit extracts the growth of *S. cerevisiae* yeast and *S. marcescens*, *P. putida* and *M. luteus* bacteria. Coban

and Biyik (2010) obtained similar results in their experiments, their studies showed an effective inhibition of *Proteus* sp., *B. cereus*, *M. luteus* and *Enterococcus faecalis* strains growth (zone of inhibition was 7–13 mm).

The most sensitive for used plant extracts among analysed microorganism was yeast of *S. cerevisiae*. Other studied fungi were generally resistant for used extracts, only lingonberry and quince fruit extracts inhibited the growth of those yeasts. The tested fungal strains proved to be sensitive to amphotericin B. *S. cerevisiae* showed approximately two-fold zone of inhibition with respect to an antibiotic in combination with other microorganisms.

The analysed bacteria were characterized by the resistance to the action of vancomycin and amoxicillin with clavulanic acid. Vancomycin and amoxicillin together with clavulanic acid showed wide spectrum of action on bacterial cultures. We found various activity of these antibiotics against tested strains. Vancomycin was characterized by the highest activity against anaerobic *Bifidobacterium* sp. and *Clostridium* sp. bacteria, which can be connected with the inhibition of peptidoglycan synthesis – the main component of the bacterial cell wall (Wilhelm and Estes, 1999). In the case of amoxicillin with clavulanic acid, the tested microorganisms were not sensitive to the bactericidal activity of this antibiotic.

In this study, a negative control accounted for 80% vol. ethanol and methanol solutions. It has been shown that the type of a solvent had a large effect on the inhibition of the growth of tested microorganisms. Analysing the microbial inhibition zone it was found that ethanol was characterized by a higher effect relative to methanol, which showed lower antimicrobial activity. Based on the obtained results it was found that the alcoholic solvent present in the plant extracts tends to hinder the growth of microorganisms compared to the neutral water.

The results of this study allow concluding that the activity of the analysed species of medicinal plants against tested microorganisms is relatively small. Experiments should be continued enabling plants to increase the efficiency of inhibition of microbial growth including usage of their therapeutic properties in combination with chemotherapeutic agents.

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ORIGINAL PAPER

Characterization of Bacteriocin-Producing Lactic Acid Bacteria Isolated from Native Fruits of Ecuadorian Amazon

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Abstract

Tropical, wild-type fruits are considered biodiverse "hotspots" of microorganisms with possible functional characteristics to be investigated. In this study, several native lactic acid bacteria (LAB) of Ecuadorian Amazon showing highly inhibitory potential were identified and characterized. Based on carbohydrate fermentation profile and 16S rRNA gene sequencing, seven strains were assigned as *Lactobacillus plantarum* and one strain as *Weissella confusa*. Using agar-well diffusion method the active synthetized components released in the neutralized and hydroxide peroxide eliminated cell-free supernatant were inhibited by proteolytic enzymes, while the activity was maintained stable after the treatment with catalase, lysozyme, α-amylase and lipase suggesting their proteinaceous nature. The inhibitory activity was stimulated by acidic conditions, upon exposure to high heat and maintained stable at different ranges of sodium chloride (4–10%). The DNA sequencing analysis confirmed the presence of *plw* structural gene encoding for plantacirin W in the selected *L. plantarum* strains. Moreover, we showed that the active peptides of Cys5-4 strains contrast effectively, in a bactericidal manner, the growth of food borne *E. coli* UTNEc1 and *Salmonella* UTNSm2, with about tree fold reduction of viable counts at the early stage of the target cell growth. The results indicated that the bacteriocin produced by selected native lactic acid bacteria strains has elevated capacity to suppress several pathogenic microorganisms implying their potential as antimicrobial agents or food preservatives.

Key words: antimicrobial substances, bacteriocins, foodborne pathogens, lactic acid bacteria (LAB)

Introduction

Worldwide, the presence of spoilage bacteria in food represents a serious problem with repercussion on consumer safety and security, therefore, identifying new alternatives for natural food preservation is of priority. Numerous studies demonstrated the capacity of probiotic microorganisms to inhibit pathogens through different mechanisms such the production of antimicrobial agents (*e.g.* bacteriocin or bacteriocin-like peptides), organic acids (*e.g.* lactic acid, hydrogen peroxidase) or throughout competing with other microbes on binding target cell sites and receptors (Danielsen and Wind, 2003; Collado *et al.*, 2007; Longdet *et al.*, 2011; O'Shea *et al.*, 2011; Zendo, 2013; da Silva Sabo *et al.*, 2014). Bacteriocins of lactic acid bacteria (LAB) are ribosomal synthetized, extracellular and antimicrobial peptides or a complex of proteins biologically active with low molecular weight that reach into the target cells by binding to their surface receptors, cell lyses, pore formation or degradation of cellular DNA (Atrih *et al.*, 2001; Corsetti *et al.*, 2004; Nes and Johnsborg, 2004; Deegan *et al.*, 2006). The LAB bacteriocin attracted significant attention for food industry due to their GRAS status (Generally Considered Safe) and potential use in bio-preservation (Reis *et al.*, 2012). As the inhibitory effect is strain related, in the last few years, the research in many laboratories was centered on identification and selection of novel lactobacilli able to produce bioactive compounds with stronger inhibitory activity against numerous foodborne pathogens to be further aggregated in aliments as preservatives (Todorov, 2008; Todorov *et al.,* 2011; Todorov *et al.*, 2013; da Silva Sabo *et al.*, 2014). In Ecuador, Provence of Imbabura, considerable human illness related to food contaminants such as salmonellosis, shigellosis *etc.* were reported by the Ministry of Health (Gaona, 2013). Most artisanal minimally processed foods, typical dishes (*i.e.* mote) and natural fruit or cereals fermented drinks (*i.e.* chicha), maintained in defective storage conditions or manipulated incorrectly appears to pose significant number of pathogens, therefore the risk of developing

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diseases is elevated. Consequently, increasing attention at the policymaker's level was assumed to increase the control and protection of the consumer by preventing contamination, improving communication about safety with producers, packers, processors and distributors by facilitating relevant research on food preservation.

Previously, we reported the probiotic potential of some lactic acid bacteria and their capacity to inhibit pathogenic microorganisms (Benavidez *et al*., 2016, Tenea and Yépez, 2016). Since the identification of microorganisms from natural sources has been considered a powerful mean for obtaining useful and genetically stable strains in this study, we explored the native fruits of Amazon rainforest with respect to identifying indigenous bacteria producers of bioactive substances with possible use as antimicrobial agents for further control and prevention of food contamination.

Experimental

Materials and Methods

Isolation and selection of LAB strains with antimicrobial activity. Fruits of native plant species: *Chrysophyllum cainito, Solanum stramofolium*, *Cheilocostus specious* and *Theobroma grandiflorum*, were initially collected from Sucumbíos rainforest (Provence located at the northeast of Ecuador, in Amazon Region), transferred into Erlenmeyer flasks (500 ml) containing sterile water (100 ml) and incubated statically for up to 5 days at room temperature. MRS agar (De Man *et al*., 1960) plates were inoculated and incubated under anaerobic conditions (37°C for 72 hours) and individual colonies were randomly selected and purified. The purified colonies (< 100 colonies/ each sample) were Gram stained, tested for mobility, indole, catalase-production, spore formation and gas production from glucose. Thirty selected LAB isolates were further screened for antimicrobial activity using agar-well diffusion assay against the following indicator strains: *E. coli* ATCC 25922, *Salmonella enterica* subsp. *enterica* ATCC 51741, *E. coli* O157:H7 UTNEc1 (isolated from local fresh cheese) and *Salmonella typhimurium* UTNSm2 (isolated from local cooked chicken) (Benavidez *et al*., 2016). Although all thirty isolates displayed antimicrobial activity against at least one indicator strain, eight strains showing broad range activity towards all indicator bacteria were further characterized for the presence of bacteriocin-like substances (BLIS).

Identification of selected LAB isolates. The carbohydrate fermentation API50CHL strips were used following the manufacturer guidelines (Biomerieux, Marcy l'Etoile France, cat # 50300). The results obtained after incubation of strips for 48 hours at 37°C were analyzed using the API software. Moreover, the bacteriocin-producing species were identified based on 16S rRNA gene sequencing (Macrogen Inc., Korea, custom-service). Briefly, the primers 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3' and 1492R 5' (TAC GGY TAC CTTGTT ACG ACT T) 3' were used for the PCR amplification. The PCR reaction was performed with 20 ng of genomic DNA in a 30 µl reaction mixture by using EF-Taq (SolGent, Korea) as follows: activation of Taq polymerase at 95°C for 2 minutes, 35 cycles of 95°C for 1 minute, 55°C and 72°C for 1 minute, finishing with 10 minutes at 72°C. The amplification products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). Sequencing reaction was performed using a PRISM BigDye Terminator v3.1 Cycle sequencing Kit. The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95°C for 5 minutes, followed by 5 minutes on ice and then analyzed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). A homology search of the sequences was conducted using BLAST program at the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST).

Antimicrobial activity. The agar-well diffusion assay was used (Benavidez *et al*., 2016). Briefly, LAB strains were grown in MRS broth at 37°C for 20 hours and the supernatants were collected by centrifugation at 13,000 × g for 20 minutes, 4°C. The CFS (crude extract supernatant) was recovered and filtered using 0.22 µm porosity syringe filter. The indicator strain (100 µl) grown in broth medium (7 log CFU/ml) were mixed with 3.5 ml of soft MRS agar (0.75%), overlaid on the nutrient agar plates and incubated at 37°C for 2 hours. The CFS of each strain (100 µl) were transferred onto the wells (6 mm) on overlaid agar, incubated at 37°C and subsequently examined for inhibition zones at different intervals of time (24–48 hours). To rule out the possible inhibition activity of organic acids, the CFS was heated at 80°C for 10 minutes, the pH adjusted at 6.0 (TFS – neutralized CFS pH 6.0) and the activity was determined. The experiments were run in triplicate and the mean value of the inhibition zone was determined. As control, *Lactobacillus plantarum* ATCC 8014 has been used.

Enzymatic sensitivity. To investigate the chemical nature of antimicrobial substances, filtered CFS were submitted to different treatments and the activity was evaluated using well-diffusion assay followed by determination of bacteriocin titer expressed as arbitrary units per ml (AU/ml). One arbitrary unit was defined as the highest dilution showing about 2 mm of inhibition zone on the indicator lawn (Todorov, 2008). Briefly, aliquots of CFS were buffered with 7% solution of calcium carbonate and the activity was determined in compari-

son with untreated CFS. When the inhibition zone was determined around the wells the inhibitory effect was assumed to be as results of bacteriocin or hydrogen peroxidase (Jimenez-Diaz *et al*., 1993). In other batch TFS was treated with catalase enzyme (1 mg/ml) to prevent the possible inhibitory of hydrogen peroxidase (NCFS – neutralized CFS and hydrogen peroxide eliminated). Moreover, NCFS was independently treated with proteinase K, trypsin, pepsin, lysozyme, α-amylase and lipase (Sigma-Aldrich Corporation, USA) at the final concentration of 1 mg/ml, incubated for 2 hours at 37°C and 5 minutes at 100°C to enzyme inactivation. All experiments were run in triplicate using *E. coli* O157:H7 UTNEc1 and *S. typhimurium* UTNSm2 as indicator strains. The control for all experiments was sterile MRS medium.

Effect of heat, pH, detergents and sodium chloride on bacteriocin activity. Aliquots of CFS were incubated for 10, 30, 60 and 75 minutes at 60, 80, 90, 100°C as well as 15 minutes at 121°C (autoclaving). In other batch, aliquots of the CFS were adjusted at the pH 2.0, 4.0, 6.0 and 10.0, incubated for 3 hours at room temperature. The effect of Triton X-100 (BDH Chemicals Ltd, Poole, England), EDTA (Merck, US), SDS (Sigma-Aldrich Corporation, US) and Tween 20 (Sigma-Aldrich Corporation, US) at the final concentration 10 mg/ml was also evaluated. Furthermore, aliquots of CFS were treated independently with 1%, 4%, 7% and 10% sodium chloride, incubated for 1 hour at 30°C. All experiments were run in triplicates using *E. coli* O157:H7 UTNEc1 and *S. typhimurium* UTNSm2 as indicator strains. The control for all experiments was sterile MRS medium.

Detection of bacteriocin encoding gene. PCR amplification of DNA genomic (PureLink™Genomic DNA minikit, #K1820-00, Invitrogen) of selected LAB was performed using published primer sequences to target plantacirin W gene (20). The primers sequences were: plwFW 5' (GCG CTT GCC AAT GAA CAA AT) 3' and plwRW 5' (TAT CTT CTC CCC AAA CTC AC) 3'. The reaction was realized in a Thermocycler device (MultiGene, Labnet International Inc.) with a Taq Platinum DNA Kit (Invitrogen) in a total volume of 50 µl. The amplification profile was as follows: 1 cycle of 5 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 52°C, and 1.5 min at 72°C; and 1 cycle of 10 min at 72°C. The amplification products (591 bp) were electrophoresed in a 1.2% agarose gel in 1X TBE (Tris-Borate-EDTA) buffer at 100 V for 1.5 h, using a 100 base-pair ladder as a fragment size marker (Trackit Invitrogen) and visualized by ethidium bromide staining. Moreover, the PCR fragments were sequenced (Macrogen Inc., Korea, custom-service). A homology search of the amino acid sequences was conducted using BLASTX program at the NCBI database (http://www.ncbi.nlm.nih.gov/BLASTX).

Effect of bacteriocin producing Cys5-4 strain on target viability. Five hundred milliliter of CFS containing bacteriocin-producing Cys5-4 was precipitated with 60% ammonium sulfate, incubated overnight at 4°C and centrifuged at 8000 × g for 30 minutes. The precipitated peptides were recovered in ammonium acetate 25 mM and stored at (–) 20°C before use. Ten milliliter of Cys5-4 bacteriocin was added to 90 ml of 3 hours old culture ($OD_{605} = 0.2$) of indicator strains (Mahrous *et al*., 2013). Incubation was performed at 37°C for 9 hours and OD_{605} was measured every hour using spectrophotometer UV-VIS (Nova60, Millipore, Merck) followed by plate-agar method to determine the viable cell counts. As control, the untreated indicator strain culture have been used. The same procedure was applied when adding the bacteriocin in the stationary-phase of the indicator cell growth.

Statistical analysis. For the effect of pH, temperature and sodium chloride treatments, the ANOVA with split-split-plot experimental design was performed. Then, Duncan's multiples range tests and LSD (Least Significant Difference with Bonferroni correction) were applied to determine significant differences between the means. The statistical significance used was $P < 0.05$ (SPSS version 10.0.6, USA and Excel).

Results and Discussion

Identification and characterization of bacteriocinogenic LAB strains. Tropical, wild-type fruits offer a new source of microorganisms with possible biotechnological characteristics to be identified. From nutritional or medicinal point of view, the consumption of those fruits is limited to the local tribes; however, the bacterial microbiota of those fruits is not well investigated. It is believed that the microorganisms from this region might provide a newly source of functional compounds to be for further exploited industrially. The lactic acid bacteria are known for extensive use as probiotics and "natural" preservatives to control harmful microorganisms in food (Georgieva *et al*., 2009; Arena *et al*., 2016). Previous studies suggested that antimicrobial components production depends on the LAB species and several exogenous factors such as medium composition, growth factors, organic and inorganic salt treatments, *etc.* (Arena *et al*., 2016). In this study, eight novel indigenous LAB strains were isolated, identified and characterized for the presence of antimicrobial substances. Based on carbohydrate profiles and confirmed by 16S rRNA sequencing, the isolates assigned Cys2-1 (GenBank accession no. KY041683), Cys4-1 (GenBank accession no. KY041685), Cys5-4 (GenBank accession no. KY041686), Cys7-1 (GenBank accession no. KY041687), Gt2 (GenBank accession

Table I
LAB identification and the residual activity (%) of neutralized cell-free supernatant

Code isolate	Species / GenBank Accession Number*	Residual activity (%)			
		E. coli UTNEc1	*S. typhimurium* UTNSm2	*E. coli* ATCC 25922	*S. enterica* subsp. *enterica* ATCC 51741
Cys2-1	*L. plantarum* / KY041683	94.78	92.67	87.80	87.80
Cys2-2	*W. confusa* / KY041684	92.30	90.26	86.95	86.95
Cys4-1	*L. plantarum* / KY041685	97.32	92.49	88.23	88.23
Cys5-4	*L. plantarum* / KY041686	97.69	86.55	90.17	90.17
Cys7-1	*L. plantarum* / KY041687	97.38	85.73	87.65	88.65
Gt2	*L. plantarum* / KY041688	97.32	94.78	85.20	85.16
Gt4	*L. plantarum* / KY041689	83.74	90.71	97.87	97.88
Gt6	*L. plantarum* / KY041690	90.26	89.67	90.18	90.18
Control	*L. plantarum* ATCC8014	92.85	89.10	88.73	88.73

* Assigned number by NCBI (https://www.ncbi.nlm.nih.gov).
The results of the assay are from three experiments, each with triplicate samples.
Residual activity in % was calculated as ratio between the inhibition zone of TFS and inhibition zone of CFS counterpart multiplied with 100.

no. KY041688), Gt4 (GenBank accession no. KY041689) and Gt6 (GenBank accession no. KY041690) were identified as *L. plantarum* and the isolate assigned Cys2-2 (GenBank accession no. KY041684) was identified as *Weissella confusa* (Table I). *L. plantarum* is known as one of the most versatile LAB species with a broad range of antimicrobial activity against Gram-positive and Gram-negative bacteria (Ali and Musleh, 2015). Recently, *W. confusa* A3 isolated from dairy products, with capacity to inhibit pathogenic bacteria as result of dextran, was reported (Goh and Philip, 2015). Although a slightly decrease in residual activity was observed when removal of organic acids the eight selected LAB strains exhibited homogeneous antimicrobial activity (above 85%) towards all four indicator strains suggesting the presence of active compounds (Table I). Besides, the organic acids in CFS, locally produced in the bacterial growth, might establish the appropriate micro environmental condition to activate the antagonistic mechanism of produced peptides against harmful microorganisms founded in the same niche. Additionally, the presence of organic acids might be beneficial if use CFS in food due to their enzymatic resistance and higher solubility compared with the bacteriocin-like peptides. The effectiveness of inhibitory activity was not influenced by the addition of calcium and removal of hydrogen peroxide (NCFS), while the treatment with proteinase K, trypsin and pepsin completely abolished the activity (Table II). Similar characteristics were observed in case of *Enterococcus mundtii* QU2, isolated from soybean, when bacteriocin production was negatively regulated albeit calcium was added to the bacterial culture medium, while treatment of crude bacterial extract with calcium the activity was still maintained (Zendo *et al.*, 2005). After lysozyme treatment the activity was maintained stable indicating that the protein might be glycosylated, while treatment with lipase and α-amylase explain the lack of carbohydrate or lipid moiety. The sensibility to enzymes might be considered an advantage in case of incorporate in food meaning that upon ingestion will not alter the digestive tract (Ali and Musleh, 2015). Overall, our data indicated the presence of antimicrobial substances (bacteriocin-like) in the bacterial crude extract of selected native LAB strains with elevated capacity to inhibit pathogenic bacteria.

Table II
Effect of enzymes and pH on bacteriocin activity of selected native LAB

Treatment		Activity (AU/ml)
Enzymes (1 mg/ml)	NCFS + Proteinase K	–
	NCFS + Trypsin	–
	NCFS + Pepsin	–
	NCFS + Lysozyme	6400
	NCFS + Lipase	6400
	NCFS+ α-amylase	6400
	NCFS	6400
	TFS	6400
pH	2.0	12800
	4.0	6400
	6.0	3200
	10.0	800
	CFS	6400

The results of the assay are from three experiments, each with triplicate samples.
CFS-crude extract supernatant; TFS-neutralized CFS (pH 6.0);
NCFS: neutralized CFS and hydrogen peroxide eliminated.

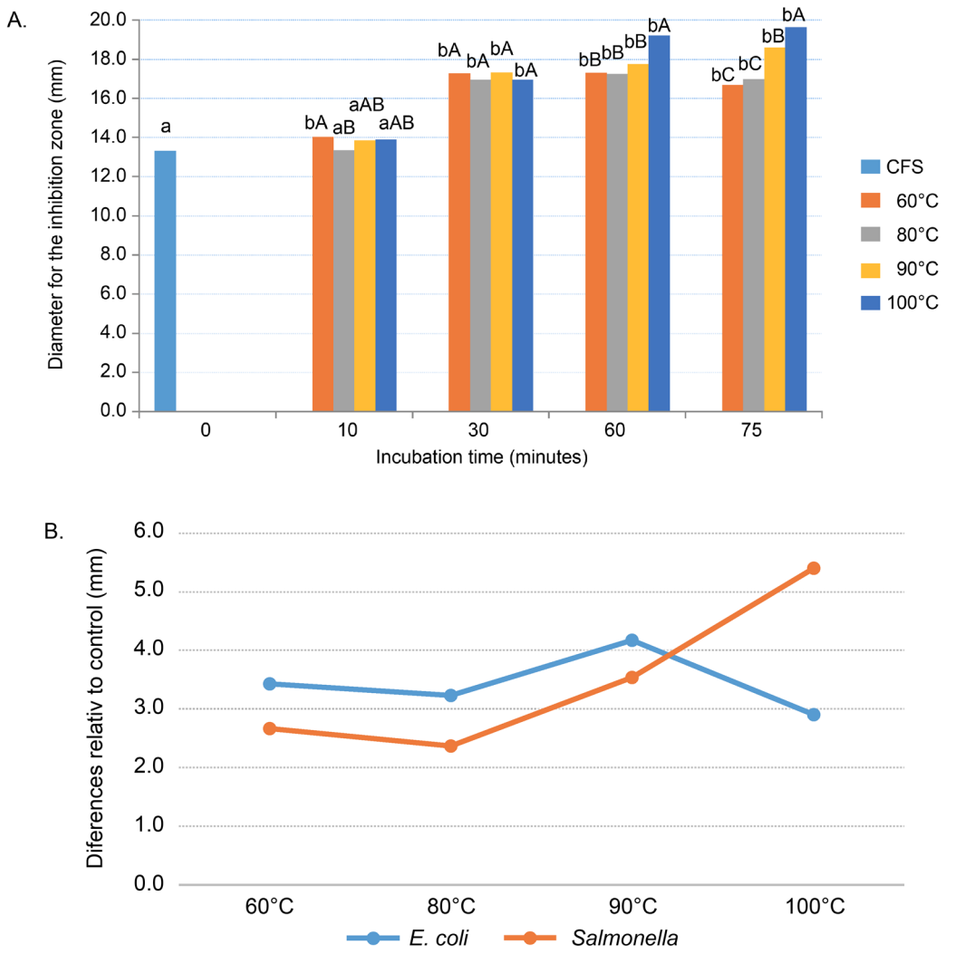

Fig. 1. Effect of heat on bacteriocin activity.

A. Diameter of the inhibition zone (mm) at different temperatures and incubation time. Bars are the means ± standard error. Values with different letters are significantly different $P < 0.05$. Small letters show the difference between temperature-incubation time and control (LSD with Bonferroni correction); Capital letters indicate the differences within incubation time (Duncan's test). B. The influence of pathogen in the inhibitory activity. The differences relative to control in the inhibition zone are shown (mm). The significant different is observed at 100°C. CFS: cell-free supernatant without incubation.

Effectiveness of inhibitory activity upon heat, pH and detergents exposure. Heat resistance is one important characteristic to be considered when selecting BLIS (bacteriocin-like inhibitory substances) producing strains to be used as preservation agent in processed food. Previous studies indicated that bacteriocins of *L. plantarum* or *W. confusa* species showed stability after heat and lower pH exposure (Goh and Philip, 2015; Arena *et al*., 2016). In this study, the statistical analysis revealed that the effectiveness of inhibitory activity was influenced by both temperature-time and pathogen-temperature interactions. Analysis from split-split-plot design (where main plot: pathogen; sub-plot 1: temperature; sub-plot 2: incubation time) indicated that the activity was maintained at the same level as untreated control after 10 minutes of incubation at all four temperatures tested, while a significant increase was recorded after 30, 60 and 75 minutes incubation (LSD with Bonferroni correction) (Fig. 1A). An increase in antimicrobial activity was recorded at the highest temperatures (90 and 100°C) and after more than 60 minutes incubation time. We hypothesized that the enhanced inhibitory activity after heat exposure throughout time might follow the same pathway as heat-treatment-induced chemical reaction between active components as amino- and carbonyl groups, known as Maillard Reaction (Hiramoto *et al*., 2004). Early studies reported the beneficial role of Maillard reaction products on pathogenic inhibition stating that high-molecular weight proteins might develop antimicrobial activity when binding chemical elements such iron, copper or zinc, known for their essential role in the growth of pathogenic microorganism (Hiramoto *et al*., 2004; Trang *et al*., 2013). Likewise, a statistically

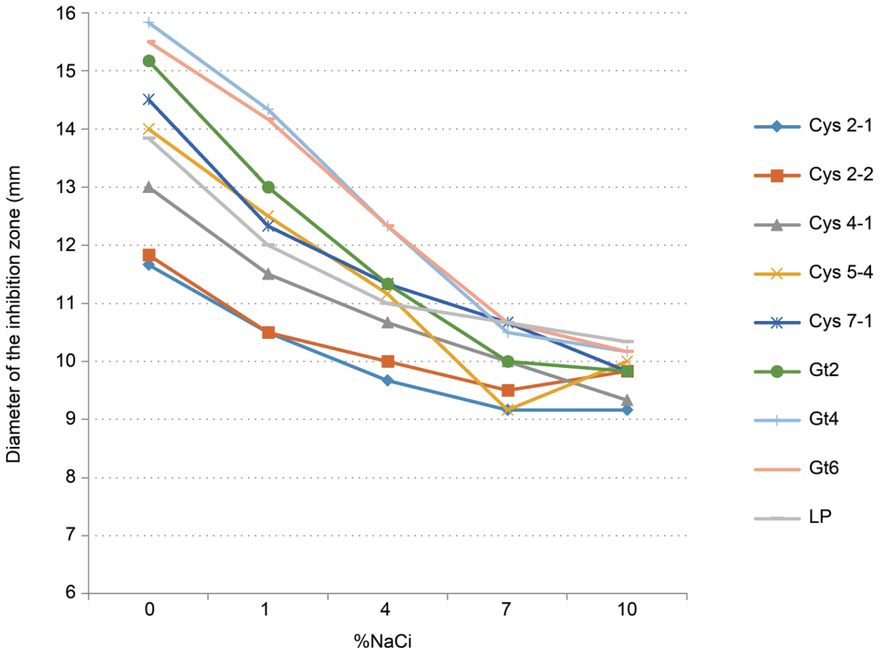

Fig. 2. Stability of bacteriocin activity upon NaCl treatment. Data represent the mean and standard error of the mean. LP: *L. plantarum* ATCC8014; 0-CFS without treatment

significant difference between both pathogens within same temperature level was observed at 100°C (Fig. 1B). Moreover, after 15 minutes autoclaving the activity remained stable for all selected LAB (*e.g.* inhibition zone of 12.00 ± 0.0 mm towards *E. coli* and 12.36 ± 0.5 mm towards *Salmonella)* indicating the advantage of those active molecules if tested as preservatives in combination with thermal processing food. Considering tropical niches origin of the raw material, this might be a significant finding as other studies did not mention such property of heat-time inducing inhibitory activity of lactic acid bacteria.

A statistically significant increase ($P < 0.05$) in activity was observed in highly acidic conditions (pH 2.0) with titer of 12800 AU/ml towards both indicator strains (Table II). At the pH 4.0 and 6.0 the titer was 6400 AU/ml while a significant decrease was register at the pH 10.0 (800 AU/ml). It is likely that effectiveness of antimicrobial activity is pH dependent. After 24 hours of incubation of bacterial culture the pH range between 3.8–4.5, however, the maximum activity was exhibited at pH 2.0. Previously, it was suggested that under acidic conditions the enhanced activity might be attributed to the increase of bacteriocin solubility or to the ability of acids to pass beyond the target cell membranes acidifying the cytoplasm and increasing its permeability, thus leading to inhibition of pathogen growth (Banerjee *et al.*, 2013). In addition, a significant increase in antimicrobial activity ($P < 0.05$) relative to untreated counterpart was observed when adding EDTA and SDS (12800 AU/ml) for all selected LAB strains. On the other hand, a slightly decrease in activity was observed when CFS was treated with Triton-X100 (3200 AU/ml), while no changes occur after treatment with Tween 20 (6400 AU/ml). As previously described, the exposure to several detergents might enhance activity through penetration of outer membrane of Gram-negative strains beyond extracting cations ($Ca^{2+}Mg^{2+}$) thus allowing bacteriocins to reach the cytoplasmic membrane (Galvez *et al.*, 2007). Alike, in this study the effectiveness of inhibitory activity was positively influenced by the treatments with SDS and chelating EDTA agent. In conclusion, our results indicated that efficiency of bacteriocin-like substances of selected LAB is positively regulated by acidic condition and heat.

BLIS-producing LAB stability at higher sodium chloride. The activity of bacteriocin produced by *L. plantarum* isolates was maintained stable after treatment with sodium chloride indicating that the inorganic salts might have a synergistic effect on bacteriocin efficacy (Fig. 2). Similar study on bacteriocin produced by *L. plantarum* LPCO10 strain showed that the activity was stable at 4% NaCl (Jimenez-Diaz *et al.*, 1993), while 10% NaCl abolished the activity (Mahrous *et al.*, 2013). In contrary, in the current study the bacteriocin activity was maintained stable in the presence of higher salt suggesting that no changes might occur in protein conformation thus demonstrating its advantage if using as preservative as interaction with higher salt content

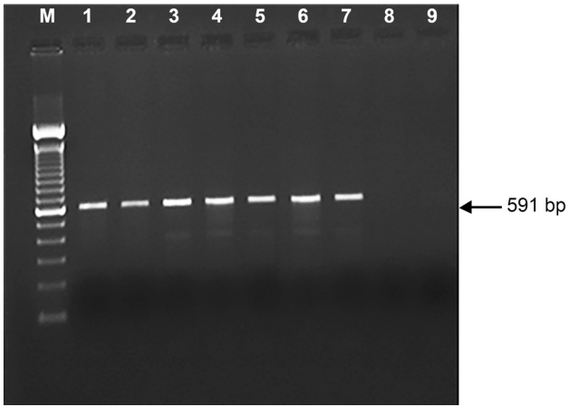

Fig. 3. Amplification fragment of 591 bp corresponding to *plw* gene detected in agarose gel electrophoresis.
M: 100 bp molecular marker, samples 1–7: DNAg isolated from *L. plantarum* isolates, 8: Cys2-2 isolate; 9-negativ control (PCR mix without DNA).

food would not have totally inhibiting their activity. Taken together, these results correlates with our previous findings about the growth tolerance of selected LAB at lower pH (2.0), in culture medium containing higher concentration of NaCl (6%) as well as high temperature of growth (40°C) (data not shown).

Detection of bacteriocin encoding *plw* structural gene. The primers used for the amplification of 591 bp fragment were complementary to sequences occurring proximal to the 3' and 5' ends targeting the structural *plw* gene (Halo *et al*., 2001). The expected fragment was identify in *L. plantarum* strains while no amplification was detected for the Cys2-2 meaning that the strain is not producing plw (Fig. 3). The nucleotide sequence homology of the selected *L. plantarum* strains except Cys4-1 was matching 100% the *plw* gene locus of *L. plantarum* RS5. The amino acid sequence analysis showed 100% identity with plantacirin W alpha and beta precursors of *L. plantarum* LMG2379. In addition, the propeptide plwα showed 41% identity with a hypothetical protein of *Streptococcus aureus* and 53% with a lantibiotic mersacidin of *Bacillus liqueniformis*. In previous study, it has been shown that both plantacirin peptides containing leader sequences, once processed gave rise to mature peptides of 32 and 29 amino acids that might act synergistically to exert their inhibitory activity (Halo *et al*., 2001). No similarity at nucleotide level with other bacteriocins has been found for Cys4-1, although the translation to amino acids sequence revealed 49% identity of with plantacirin W beta precursor.

Effect of Cys5-4 bacteriocin on indicator cells viability. Bacteriocins act as bactericidal or bacteriostatic manner depending on the dose, degree of purification and the physiological stage of the growth of indicator cells. In this study, the addition of bacteriocin at the early stage of growth resulted in decreasing of the target cell viability after 2 hours of incubation and maintained up to 9 hours of measurement suggesting that the Cys5-4 bacteriocin might follow a bactericidal pathway. The viability of *E. coli* decreased from 6.16 to 2.32 log CFU/ml (Fig. 4) and from 6.28 to 3.53 log CFU/ml, respectively, for *Salmonella*. Similarly, when the bacteriocin was added at the late phase (OD 0.7) the viability was reduced but not at the same level as in the early stage of target growth (data not shown). This complies with early studies, which revealed that bacteriocin-like inhibitory substances were active during the exponential to early stationary phase of growth (Hernandez *et al*., 2005). The indicator cells were smaller than those seen in the positive control cells suggesting that the active bacteriocin Cys5-4 might bind the cell-wall leading to their destabilization, thus exerting its bactericidal mode of action. Those findings must be validated *ex vitro* knowing that the food act as complex ecosystem in which interactions between active components with target cells and other metabolic components of food matrix might influence the inhibitory mechanism.

While several studies described numerous lactobacilli isolated from human, animal or vegetable with antimicrobial activity (Zambou *et al*., 2013), up to now,

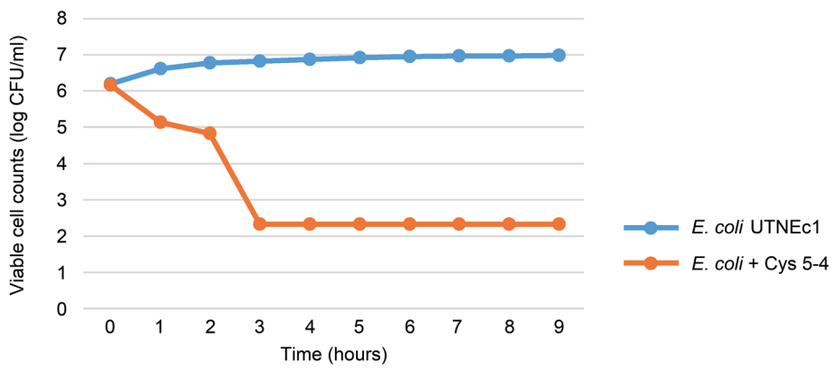

Fig. 4. Effect of bacteriocin Cys5-4 on the viability of *E. coli* UTNEc1.

no study about the presence of bacteriocinogenic LAB in native fruits of Ecuadorian Amazon rainforest was shown. Early investigation on *L. plantarum* PMU33 strain isolated from a fermented fish food reported the presence of two peptide bacteriocin plwα and plwβ with tolerance to heat and acidity (Noonpakdee *et al*., 2009). Here, we showed that the bacteriocin activity of selected strains is enhanced upon heat exposure throughout time demonstrating their valuable potential as used in processed food. Furthermore, we reported for the first time the presence of a native *W. confusa* and *L. plantarum* strains that produced inhibitory substances with strongest capacity to suppress the growth of foodborne pathogens founded in the local market implying further investigation to demonstrate their promising potential as antimicrobial agents.

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Conflict of interest

The authors declare that they have no conflict of interest in the present publication.

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ORIGINAL PAPER

Physiological Effects of Whey- and Milk-Based Probiotic Yogurt in Rats

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Abstract

In an *in vitro* experiment commercially available probiotic products were tested for the survival of bacteria under conditions of simulated human digestion either when used alone or mixed into yogurt. In the *in vivo* experiment the effects of feeding a whey- and milk-based yogurt prepared with the probiotic strain showing adequate survival in the *in vitro* experiment, was measured on body weight, feed consumption and immune response of rats (IgG and IgA level after immunisation), on the composition and volatile fatty acid production of the intestinal microbiota and on the structure of intestinal villi. The *Lactobacillus acidophilus* (LA-15) strain had inadequate surviving ability in rats. *Bifidobacterium animalis* ssp. *lactis* (BB-12) improved the composition of the intestinal microflora, whereas whey-containing product had a mild immunostimulating effect and exerted a favourable influence on the morphology of intestinal villi. The consumption of yogurts increased the depth of crypts in the ileum, which resulted in enhanced secretion and thus softer faeces.

Key words: *Bifidobacterium animalis*, *Lactobacillus acidophilus,* rat intestine physiology, physiological effect of whey and yogurt

Introduction

Probiotics are live microorganisms which, when administered in sufficient quantities, exert a favourable effect on health status (FAO/WHO, 2002). Numerous bacterial species have been suggested to have probiotic effects, but lactic acid producing bacteria (*Lactobacillus*, *Bifidobacterium*, *Streptococcus*), which occur in the body naturally as constituents of the healthy gut microbiota, have gained the widest use in the practice. Some supposed and confirmed effects through which probiotic microbes exert their favourable effects in the intestine are: supporting the mucosal barrier function of the gut, production of antimicrobial substances (*e.g.* bacteriocins), pH reduction, colonisation of the surface of the intestinal epithelium (competitive exclusion), immunostimulatory effects, anti-inflammatory properties (Kumar *et al*., 2015). The proportion of fermented dairy products is increasing steadily on the market of probiotic foods (Özer and Kirmaci, 2010). Commercially available dairy products often contain specific strains of the genera *Lactobacillus* and *Bifidobacterium* that meet the requirements listed above (Gueimonde *et al*., 2004).

Environmentally conscious production is important during milk processing. The disposal of by-products arising in large quantities (including whey, which is the portion of milk that is left behind after removing the casein protein) raises environmental concerns (Chatzipaschali and Stamatis, 2012).

Earlier, whey was used only for animal nutrition, but it can be added to dairy products at a rate of 30–75% without reducing the enjoyment value of the products (Castro *et al.* 2012). The consumption of whey-based products is increasing all over the world (Boynton and Novakovic, 2014). This by-product, which makes up 85–95% of milk, contains valuable substances as it includes 55% of the nutrient content of milk: lactose (45–50 g/l), soluble proteins (6–8 g/l), fats (4–5 g/l), and minerals (8–10% of the dry extract) (Farizoglu *et al*., 2004).

The objective of this series of experiments was to select effective probiotic strains for the development of a whey-based dairy product. In addition, we compared the physiological effects of whey- and milkbased products as well as of probiotic and non-probiotic products in rats.

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Experimental

Materials and Methods

***In vitro* experiment.** Cultures containing *Bifidobacterium animalis* ssp. *lactis*, or *Lactobacillus acidophilus*, or combination of probiotic strains (*Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*) were tested. A 10 ml suspension from each of the probiotic strains was prepared and the initial germ count was adjusted to 10^8 colony forming units (CFU)/ml. The survival of the probiotic strains was tested in their original condition and subsequently after mixing them into a yogurt product.

Pasteurized raw material (milk or a 50:50% mixture of milk and whey) was inoculated with the yogurt culture (0.3%) and the propionic strain. Maturation of the product was done at a temperature of 39–42°C and a pH of 4.6–4.8. After the end of the maturation process the yogurts were placed into a blast chiller for 10–16 hours, and subsequently stored in a room of less than 10°C temperature until delivery.

The *in vitro* simulated human digestion process was modelled according to the method of Versantvoort *et al.* (2005).

Study of the physiological effects of milk- and whey-based yogurt made with the selected probiotic strains in rats. Three yogurt products were studied: amilk-based probiotic (MP), a milk-based non-probiotic product (M), and a probiotic one made from the 1:1 mixture of raw milk and whey (WP). The milk was pasteurized at 85–90°C, the fat content was adjusted to 1.5% and then the raw material was cooled down to 40–42°C. The yogurt cultures were added at that temperature (FVV121 culture, 0.01%; *L. acidophilus* culture, 0.01%) during gentle mixing for 5 minutes. Warm maturation was conducted at 38–42°C for 4–5 hours, until the pH reached a value of 4.4. This was followed by cold maturation for 24 hours, during which the temperature of the yogurt was reduced below 10°C.

The probiotic product was made from two commercially available products containing *B. animalis* ssp. *lactis* (BB-12) and *L. acidophilus* (LA-15) strains, respectively.

The yogurts were stored in a refrigerator at +4°C temperature, and their germ counts were checked weekly.

A total of 40 mature male rats (Wistar Crl: WI BR, SPF, Budapest) were housed in individual cages (Techniplast, Buguggiate, Italy) and fed a commercially available rat diet (S8106-S011, ssniff Spezialdiäten GmbH, Soest, Germany) *ad libitum*. Three groups of animals (M, MP, WP, n = 8/group) received yogurt in addition to the rat diet, while the control animals (C, n = 16) were fed only the rat diet throughout the experiment. The rats fed yogurt were offered rat food *ad libitum* during the day, then at 08:00 p.m. the feeders and drinkers were closed down. A 10 ml volume of yogurt was filled into the drinkers and offered to the rats between 08:00 and 09:00 a.m., during which time the control rats were offered water.

On days 1 and 14 of the experiment the rats were inoculated intraperitoneally with 100 µg ovalbumin (OVA; Sigma-Aldrich, Hungary) per animal. Blood samples were taken before vaccination (on day 0), and subsequently on days 14 and 28. Half of the control rats were immunized (Group CI, n = 8), while the other 8 control rats did not receive immunization (Group C, n = 8).

The rats were weighed weekly. Daily feed and yogurt consumption was recorded individually.

At the end of the experiment (on day 28) the rats were decapitated and bled after narcosis with carbon dioxide. Samples were taken from the small intestine and the caecum.

The experimental protocol was authorized by the Food Chain Safety and Animal Health Directorate of the Somogy County Agricultural Office, under permission number RH/162-2/2013.

Parameters examined. Immunoglobulin G (IgG) and immunoglobulin A (IgA) were determined from the blood plasma and the saliva, respectively, on days 0, 14 and 28. For specific IgG detection, microtiter plates (Sigma-Aldrich, Hungary) were coated with 100 µl of OVA solution per each well. Aliquots of 100 µl of two dilutions (1:50 and 1:100) were transferred to the microtiter plate and incubated for 1 hour at room temperature. Wells were washed 3 times with PBS-Tween, and then anti-rat IgG-HRP conjugate (Sigma-Aldrich, 1:10,000) was added to each well, followed by incubation at room temperature for 1 hour. Total secretory IgA (sIgA) from the saliva was measured using a quantitative Rat IgA ELISA kit (Kamiya Biomedical Co., USA) as described by the manufacturer.

From 1 g of small intestinal and caecal content, serial dilutions with 0.9% sterile saline solution were made immediately after sampling and used for microbiological determination. Media used and conditions of incubation are summarised in Table I. Incubation was done under aerobic (Memmert 108 incubator, Memmert, Schwabach, Germany) or anaerobic conditions (Anaerob Jar, Merck, Darmstadt, Germany). Results were expressed in log10 CFU related to 1 g of sample.

If the probiotic strains could be detected from the small intestinal and caecal chyme pure cultures were obtained from the *Lactobacillus* and *Bifidobacterium* cultures by a single passage on the surface of the above-mentioned selective media and studied by molecular genetic tools. Partial sequences of the 16S rRNA coding gene of colonies picked up from pure cultures were determined by the use of universal bacterial primers (M13F-27F: TGTAAAACGACGGC-

Table I
Microbiological cultivation methods

Bacteria	Media	Conditions of incubation
In vitro experiment		
B. animalis ssp. *lactis*	TOS-propionate agar medium[1] supplemented with lithium-mupirocin	37°C, 72 h, anaerobic
L. acidophilus	MRS agar	35°C, 72 h, anaerobic
L. delbrueckii ssp. *bulgaricus*	MRS agar	35°C, 72 h, anaerobic
Streptococcus thermophilus	M-17 agar supplemented with 10% lactose solution[1]	42°C, 24 h, anaerobic
In vivo experiment		
Total aerobic bacteria	blood agar	30°C, 72 h, aerobic
Total anaerobic bacteria	blood agar	37°C, 48 h, anaerobic
E. coli	Chromocult differentiation medium[2]	37°C, 24 h, aerobic
Bifidobacterium	TOS-propionate agar medium[1]	37°C, 72 h, anaerobic
Bacteroides	Schaedler's agar[3] supplemented with esculin[2], neomycin[2] and iron ammonium citrate[3]	37°C, 96 h, anaerobic
Lactobacillus sp.	MRS agar[3]	37°C, 72 h, anaerobic

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CAGTCAGTC-AGAGTTTGATYMTGGCTCAG and M13R-338R: CAGGAAACAGCTATGACCCAT-GCTGCCTCC CGTAGGAGT). The 5' ends of the universal bacterial primers had M13 sequences to facilitate the sequencing reaction described later. A colony was directly used in a PCR reaction. After amplification (96°C, 5 min; 35 cycles of 94°C 30 sec, 62°C 45 sec, 68°C 45 sec followed by 72°C 2 min), the PCR product was checked on 4% MetaPhor™ (Lonza, USA) agarose gel (6 min 6 V cm^{-1}, 8 min 9 V cm^{-1}). Successful PCR reactions were treated with ExoSAP-IT™ (USB, USA) at 37°C for 15 min and at 80°C for 15 min to inactivate unconsumed dNTPs, primers and ExoSAP itself. This mixture was directly used in the sequencing reaction (Big Dye Direct Sequencing kit, Life Technologies, USA). Conditions of the sequencing reaction were: 37°C 15 min, 80°C 2 min, 96°C 1 min; 25 cycles 96°C 10 sec, 50°C 5 sec, 60°C 4 min. Primers were complements of M13 sequences presented on the 5' regions of the previously described M13F-27F and M13R-338R primers. The product of the sequencing reaction was purified by BigDye XTerminator™ (Life Technologies, USA). The sequence was determined on an ABI-3500 fragment analyser. Sequences were identified on the basis of a public 16S ribosomal RNA sequence database (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_SPEC=WGS&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch).

The concentration of volatile fatty acids (VFA) was measured by gas chromatography (Shimadzu GC 2010, Japan; Nukol 30 m × 0.25 mm × 0.25 µm capillary column, Supelco, Bellefonte, PA, USA; FID detector, 1:50 split ratio, 1 µl injected volume, helium 0.84 ml /min^{-1}). Detector conditions: air 400 ml/min, hydrogen 47 ml/min, temperature: injector 250°C, detector 250°C, column 150°C). 2-ethyl-butyrate (FLUKA Chemie GmbH, Buchs, Switzerland) was used as internal standard.

Samples for histological examination were taken from the duodenum (1 cm distal to the pylorus), the proximal part of the jejunum and the ileum (1 cm proximal to the ileocaecal valve opening). The samples were fixed in 10% buffered formaldehyde solution then embedded in paraffin and stained with haematoxylin and eosin. The general condition of the intestinal mucosa, the epithelial layer covering the intestinal villi, the structure of the *stratum villosum*, quantitative and qualitative composition of the cells were evaluated. The cytomorphology of the gut-associated lymphoid tissue (GALT) in the ileum was also examined. The length of the intestinal villi and the depth of the intestinal glands (crypts) were measured by a histometric method (using an ocular micrometer).

The effect of treatment or treatment and age was determined by one-way and multi-factor analysis of variance, respectively (SPSS 10.0, 2002). The significance of between-group differences was tested by the LSD *post-hoc* test.

Results and Discussion

Survival of the probiotic strains after *in vitro* simulated digestion. Probiotic bacterial strains could be cultured in a germ count of 10^6–10^7 magnitude, with the *B. animalis* ssp. *lactis* strains having the highest

initial germ count. After *in vitro* digestion the colony-forming unit counts of the *B. animalis* ssp. *lactis* bacteria decreased by 4 and 5 logs. The *L. acidophilus*, *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* strains survived the conditions of simulated digestion in very low numbers (only 10^1 CFU/ml).

In the yogurt the initial germ count of probiotic strains was 10^8–10^9, during the digestion the germ counts decreased by 2–6 logs.

Selection and testing of the appropriate probiotic strains require extreme circumspection. These bacteria have to survive the bactericidal effects occurring in the stomach and small intestine (gastric acid, pH, bile salts, proteolytic enzymes) in order to colonize the small or the large intestine. For this reason, it is not enough to test whether the probiotic strain can be cultured from a given product, since – as shown by our results – the germ count can decrease significantly even after an *in vitro* simulated digestion. The type and chemical composition of the food raw material selected for the production of probiotic products have a decisive influence on colonization of the gut by these bacteria. Yogurt has proved to be an ideal material (Ranadheera *et al*., 2010). This has been demonstrated also by our experiment, as the probiotic strains mixed in yogurt survived *in vitro* digestion in germ counts of 10^3–10^6, whereas their survival in their original condition was minimal: lactobacilli and bifidobacteria could be cultured only in germ counts as low as 10 to 1000.

Probiotic microbial strains used in combination may exert their beneficial effect more effectively than a single strain used alone (Timmerman *et al*., 2004). This effect probably occurs with bacteria capable of complementing the metabolism of one another, and is manifested also in the higher germ count of bacteria surviving digestion. In this experiment, in the yogurt product containing both the *Lactobacillus delbrueckii* ssp. *bulgaricus* and the *Streptococcus thermophilus* strains the bacteria survived the digestion procedure only in a germ count of 10^3–10^4.

Despite the fact that some strains of the genus *Bifidobacterium* can survive in yogurt for a short period of time (Roy, 2005), *Bifidobacterium animalis* ssp. *lactis* was the bacterium that survived the simulated human digestion procedure in the highest germ count (5.3×10^7). As we obtained favourable survival results (10^6 CFU/ml) for *L. acidophilus* strains as well, we decided on the combined use of *L. acidophilus* and *B. animalis* ssp. *lactis* strains in the rat experiment. Strains belonging to the genera *Lactobacillus* and *Bifidobacterium* are often included in commercially available dairy products (Masco *et al*., 2005).

Effects of the milk- and whey-based yogurt prepared with the selected probiotic strains in rats. The germ count of probiotic yogurts was 4×10^8 ml^{-1} for

Table II

Body weight (g), solid feed (g) and yogurt (ml) consumption of the animals (means ±SD)

Parameter	Group[1]				Date				P		
	CI	WP	MP	M	week 1	week 2	week 3	week 4	group	date	group × date
Body weight	398 ± 23	410 ± 39	411 ± 38	403 ± 38	365 ± 16^{A}	391±21^{B}	436 ± 25^{C}	445 ± 28^{C}	NS	< 0.05	NS
Solid feed consumption	27.8 ± 4.6	28.6 ± 4.9	28.6 ± 5.2	28.8 ± 4.9	28.0 ± 4.9	30.0 ± 6.1	26.4 ± 5.4	28.0 ± 4.1	NS	NS	NS
Yogurt consumption	0	5.8 ± 3.9^{b}	4.1 ± 3.0^{a}	4.2 ± 3.6^{a}	4.3 ± 2.9^{A}	5.0 ± 3.9^{AB}	5.3 ± 3.9^{B}	5.1 ± 3.4^{AB}	< 0.05	< 0.05	NS
Total consumption2	28.3 ± 4.4^{a}	34.0 ± 6.3^{b}	32.9 ± 5.8^{b}	32.8 ± 6^{b}	30.9 ± 6.1	33.0 ± 7.2	30.2 ± 7	31.4 ± 6.3	< 0.05	NS	NS

Different indices mean significant difference between ^{a,b} groups and ^{A,B,C} dates.

[1] n = 8 in each group

[2] total consumption means solid feed + yogurt intake (g)

L. acidophilus and 1.6×10^8 for *B. animalis* ssp. *lactis*, and storage at +4°C did not decrease the viability of probiotic bacteria. The rats drank about 3 – 8 ml of yogurt each, which represented an average daily yogurt intake of 2 dl for a human of average body weight (60 kg). The result of testing under *in vitro* conditions does not necessarily mean the survival of the probiotic strain in the living organism. In our animal experiment from the small and large intestinal samples of rats of groups WP and MP the *B. animalis* ssp. *lactis* (YIT4121) strain administered as a probiotic could be identified, whereas the *L. acidophilus* (ATCC 700396) strain also used as a probiotic could not be detected by molecular genetic methods. The poor survival of lactobacilli was indicated by the bacterial counts cultured from the small and the large intestine, which did not differ significantly according to whether probiotic (WP, MP) or non-probiotic (M) yogurt was fed (see later). Survival may be different under *in vitro* and *in vivo* conditions, as in the living organism the survival of bacteria is influenced also by factors as the interaction between the probiotic strains and the natural intestinal microbiota, the antimicrobial substances produced by microbes, the motility of the intestine and the local immune response (Holzapfel and Schillinger, 2002).

As the results suggested that probiotic lactobacilli were not present in the small and large intestine in sufficient numbers to exert such effects, the physiological effects found in the experiment can be attributed to bifidobacteria.

Body weight, diet and yogurt drink consumption. The body weight of the experimental animals increased approximately by 20% during the four weeks of the experiment, and there was no significant difference between the groups (Table II). Although the rats consuming the probiotic product weighed about 10 g more, the difference was not statistically significant. Yogurt consumption increased by the second week and remained roughly on the same level subsequently. The whey-containing probiotic yogurt was consumed by the rats in the highest quantity. Adding up the average daily feed and yogurt consumption of the rats, it can be seen that this was substantially higher than the feed consumption of the control rats, which means that the experimental rats consumed the yogurt in addition to the feed that they ate in the same amount as the control rats.

Immune response. There was no difference between the treatments in specific IgG level at the end of the experiment (Fig. 1A). It can be seen, however, that in rats fed the whey-containing probiotic product the IgG level increased faster and was significantly higher than in the rats fed the non-probiotic milk-based yogurt. Orally ingested whey had a more expressed beneficial effect on the local immune response as sIgA concentration of the saliva on day 14 was significantly higher in WP rats than in rats of all the other groups (Fig. 1B). Enzymatic degradation of the whey proteins results in the formation of numerous peptides of immunomodulatory effect, which have been shown to increase the concentration of specific IgG and intestinal anti-CT IgA after immunization of mice (Gauthier *et al*., 2006). In the present experiment, although the concentration of the tested antibodies increased, the consumption of neither milk nor whey had a detectable influence on the quantitative and qualitative composition of the lymphoid tissue (GALT) of the intestinal mucosa.

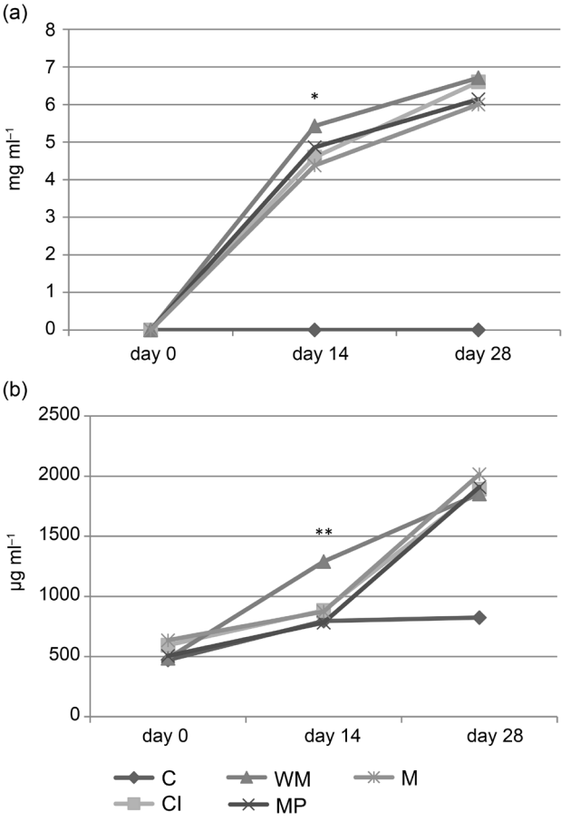

Fig. 1A. Level of OVA-specific IgG measured in the blood plasma and Fig. 1B. sIgA measured in the saliva 14 days after the first (on day 0) and second (day 14) immunisation

*WP significantly different from M

*WP significantly different from all other groups

Microbiota and fermentation. One of the main beneficial digestive-physiological effects of probiotics is stabilization of intestinal eubiosis. As had been expected, in our experiment the probiotic dairy products (WP and MP) significantly increased the counts of bifidobacteria in the large intestine, while in the small intestine a significant effect was exerted only by the milk-based yogurt (MP) (Table III). In addition to the also important Bacteroidetes (usually making up about 10–50% of the flora) and Firmicutes strains (accounting

Table III
Composition of the small intestinal and caecal microbiota (log_{10} CFU[1]/g), pH, dry matter and VFA content of the caecal chyme (means ± SD)

	CI	WP	MP	M
Small intestine				
Aerobic bacteria	7.92 ± 0.56	8.05 ± 0.09	8.04 ± 0.19	8.07 ± 0.45
Anaerobic bacteria	8.69 ± 0.33	9.05 ± 0.15	8.78 ± 0.20	8.87 ± 0.41
E. coli	4.99 ± 0.66	5.77 ± 0.66	4.82 ± 0.35	5.25 ± 0.56
Bifidobacterium sp.	3.31 ± 0.47^{a}	4.38 ± 0.59^{ab}	4.74 ± 0.40^{b}	4.38 ± 0.64^{ab}
Lactobacillus sp.	8.35 ± 0.16	8.56 ± 0.13	8.28 ± 0.18	8.31 ± 0.24
Ceacum				
pH value	6.35 ± 0.20	6.4 ± 0.1	6.24 ± 0.1	6.5 ± 0.2
Dry matter %	24.1 ± 2.2^{b}	22.4 ± 1.4^{ab}	21.9 ± 0.9^{ab}	21.0 ± 1.5^{a}
Aerobic bacteria	9.07 ± 0.21	9.58 ± 0.21	9.20 ± 0.36	9.21 ± 0.42
Anaerobic bacteria	9.78 ± 0.06^{a}	10.4 ± 0.13^{b}	10.2 ± 0.08^{b}	10.2 ± 0.31^{b}
E. coli	6.66 ± 0.45	6.67 ± 0.39	6.66 ± 0.33	7.24 ± 0.52
Bifidobacterium sp.	5.61 ± 0.21^{a}	7.40 ± 0.41^{b}	7.21 ± 0.66^{a}	6.38 ± 0.97^{ab}
Bacteroides sp.	8.51 ± 0.54	8.82 ± 0.31	8.47 ± 0.54	8.76 ± 0.53
Lactobacillus sp.	8.89 ± 0.16^{a}	9.34 ± 0.21^{b}	9.37 ± 0.22^{b}	9.28 ± 0.25^{b}
Total VFA (mmol/kg)	87.7 ± 26.1	96.9 ± 28.0	78.5 ± 17.1	98.6 ± 37.6
Acetic acid (%)	57.1 ± 1.2	61.1 ± 5.8	56.5 ± 5.4	64.1 ± 3.2
Propionic acid (%)	10.1 ± 1.2^{b}	11.6 ± 2.8^{bc}	13.0 ± 1.4^{c}	6.2 ± 0.2^{a}
Butyric acid (%)	32.6 ± 2.1	27.3 ± 3.9	30.4 ± 1.8	29.1 ± 3.5

n = 8/group; [1] CFU = colony forming unit; [a, b, c] significant difference between groups ($P < 0.05$)

for as much as 75%), *Bifidobacterium* strains constitute about 10% of the large intestinal microflora. These microorganisms have numerous favourable physiological effects; their exopolysaccharides facilitate the survival of bacteria in the gastrointestinal tract, they favourably influence the composition of the microbiota and the metabolic processes of microorganisms, and may have a beneficial effect on the immune response (Scott *et al.,* 2014). The dairy products did not have an effect on the counts of lactobacilli in the small intestine, while they increased it in the large intestine, irrespective of the probiotic *Bifidobacterium* content. Milk (M) increased the propagation of *E. coli* in the large intestine ($P > 0.05$), which was decreased by the probiotic (WP, MP), and thus in these animals an *E. coli* count similar to that found in the control rats not consuming the dairy product was demonstrated. Because of their easily fermentable lactose content, milk and whey serve as ideal substrates for lactobacilli and *E. coli*, while the other bifidobacteria and lactobacilli exert a competitive antagonistic effect on *E. coli*.

Yogurt-fed rats had slightly softer faeces containing less dry matter, but the difference from the control was statistically significant only for rats of group M (Table II). Within the total quantity of the volatile fatty acids (VFA), the proportion of propionic acid was the lowest in group M and the highest in group MP.

Bifidobacteria produce acetic acid and lactic acid in a ratio of 3:2; however, the increase in their colony-forming unit counts as compared to the control did not have a notable effect on the quantity and proportion of the volatile fatty acids produced. Only the non-probiotic milk-based yogurt resulted in a low propionic acid production. The lower water content of the intestinal content in the yogurt-consuming animals (7–15% as compared to the control) is consistent with the results obtained by other researchers: the feeding of a lactobacillus-containing probiotic increased the water content of the faeces by 7–20% in rats (Wang *et al*., 2009). In the present experiment, this could be observed also in the rats fed a non-probiotic yogurt (group M), which is possibly attributable to the fact that the number of colony-forming lactobacilli was significantly higher in these animals as well. Increased water secretion may also be attributable to the increased depth of crypts between the intestinal villi (see later), as these results in an increase in the number of secretory cells located in the crypts, leading to enhanced electrolyte and water excretion (Nabuurs and Hoogendoorn, 1993). Despite the laxative effect, the rats did not show signs of diarrhoea.

Histology. The intestinal mucosa of rats receiving different treatments showed a normal histological picture characteristic of the species and the age, with no

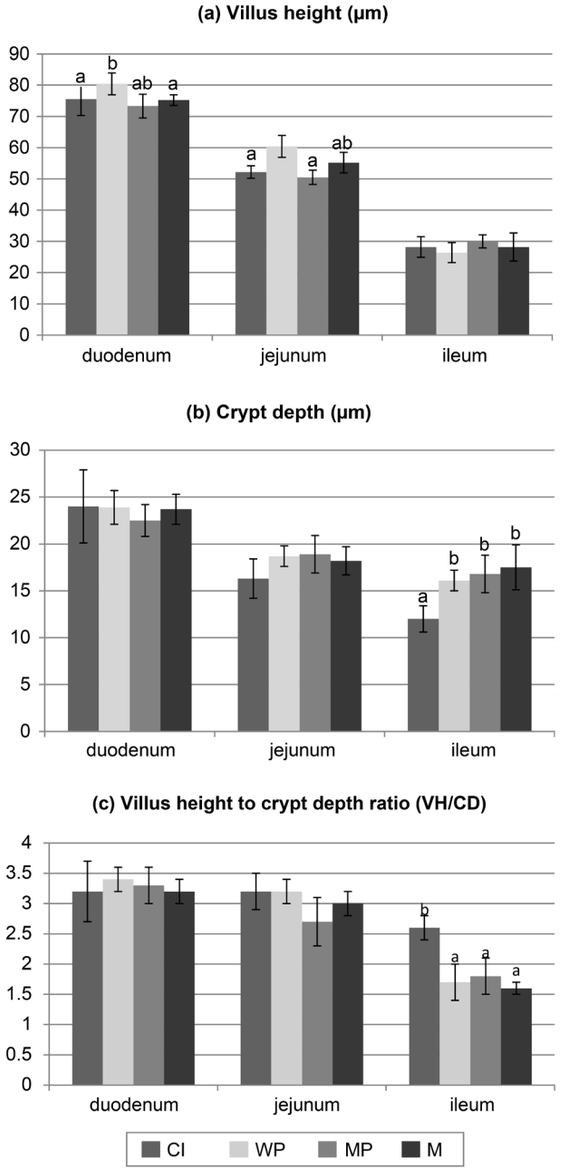

Fig. 2A. Villus height (μm), Fig. 2B. crypt depth (μm) and Fig. 2C. their ratio (VH/CD) along the small intestine.
[a, b] different indices mean significant difference between groups ($P < 0.05$)

difference in the quantitative and qualitative composition of the lymphoid tissue of the intestinal mucosa (GALT). The intestinal villi of the duodenum and jejunum were higher in group WP as compared to the other three treatments, but this did not cause a significant deviation in the calculated villus height/crypt depth (VH/CD) value (Fig. 2). The crypts in the ileum of control rats were significantly shallower, which resulted in a significantly higher VH/CD value as compared to the rats consuming yogurt.

Presumably owing to the bioactive peptides contained by it, milk whey resulted in higher intestinal villi in the duodenum and jejunum. This is indicative of a more intensive proliferation or a slower apoptosis, and results in a larger absorptive surface and higher brush border enzyme activity (Pluske *et al.*, 1996). In weaned piglets challenged with lipopolysaccharide, Xiao *et al.* (2016) studied the effect of a whey protein concentrate (WPC) on intestinal integrity, and found a greater intestinal VH and, in association with the former, a better intestinal barrier function.

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Conflict of interest

The authors declare that they have no conflict of interest.

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ORIGINAL PAPER

Identification of *Lactobacillus delbrueckii* and *Streptococcus thermophilus* Strains Present in Artisanal Raw Cow Milk Cheese Using Real-time PCR and Classic Plate Count Methods

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Abstract

The aim of this paper was to detect *Lactobacillus delbrueckii* and *Streptococcus thermophilus* using real-time quantitative PCR assay in 7-day ripening cheese produced from unpasteurised milk. Real-time quantitative PCR assays were designed to identify and enumerate the chosen species of lactic acid bacteria (LAB) in ripened cheese. The results of molecular quantification and classic bacterial enumeration showed a high level of similarity proving that DNA extraction was carried out in a proper way and that genomic DNA solutions were free of PCR inhibitors. These methods revealed the presence of *L. delbrueckii* and *S. thermophilus*. The real-time PCR enabled quantification with a detection of 10^1–10^3 CFU/g of product. qPCR-standard curves were linear over seven log units down to 10^1 copies per reaction; efficiencies ranged from 77.9% to 93.6%. Cheese samples were analysed with plate count method and qPCR in parallel. Compared with the classic plate count method, the newly developed qPCR method provided faster and species specific identification of two dairy LAB and yielded comparable quantitative results.

Key words: *Lactobacillus delbrueckii*, *Streptococcus thermophilus*, artisanal cheese microflora, LAB identification

Introduction

There is a variety of numerous microorganisms including bacteria, yeasts and moulds,

which constitute cheese microflora and form a complex microbial ecosystem. Lactic acid bacteria (LAB) contribute to forming the desirable aroma and flavour of ripened cheese. They come directly from cow milk and from an environment having contact with milk and cheese curd during manufacture and ripening. Among LAB, different genera including *Lactobacillus, Streptococcus, Enterococcus* and *Leuconostoc* participate in the cheese production (Andrighetto *et al.*, 2002; Aquilanti *et al.*, 2007a; Aquilanti *et al.*, 2007b; Blaiotta *et al.*, 2008; Callon *et al.*, 2004; García *et al.*, 2002; Mannu *et al.*, 1999). Cheeses which are produced from unpasteurized milk using traditional manufacturing procedures may contain a very differentiated microflora (Cronin *et al.*, 2007). Such biodiversity may possess a significant impact on the maintenance of the typical features of traditional cheese products (Bizzarro *et al.*, 2000; Suzzi *et al.,* 2000). In fact, recent investigations have proved that the native microorganisms present in raw milk and the ones coming from the environment are known to contribute to most of the physico-chemical and aromatic transformations taking place during cheese production (Baruzzi *et al.*, 2000; Baruzzi *et al.*, 2002; De Angelis *et al.*, 2001; Dolci *et al.*, 2008a; Dolci *et al.*, 2008b). Artisanal cheese is produced by traditional techniques using unpasteurised milk and without adding any commercial starters which are deliberately added in case of cheese produced from pasteurised milk (Albenzio *et al.*, 2001; Corroler *et al.*, 1998; Terzic-Vidojevic *et al.*, 2007). This cheese ripens at 4°C and is produced in the Podlaskie region of north-eastern Poland. The microflora originally present in raw milk is responsible for ripening of this cheese as no starters are added (Delgado and Mayo, 2004; Delbès *et al.*, 2007, Duthoit *et al.*, 2003). All the phases of manufacturing are carried out in a manual way. This distinctive cheese is closely connected with the territory of production and its tradition which are unique due to its historical and cultural environment.

Microflora of cheese usually comprise lactic acid bacteria which highly influence the human health and nutrition. They are responsible for both spontaneous fermentations and large-scale fermentation processes finding their application in the preservation as well as

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the transformation of many raw food materials such as milk, meat, fish, cereals, tubers and vegetables. LAB are also known to be commensal inhabitants of the gastrointestinal tract in humans and animals ,in which they are responsible for the complex interactions between the intestinal microbiota and the host. Among LAB, the probiotic species can be identified as they possess a significantly beneficial influence on preventing and treating diarrhoea, improving the digestion of lactose by lactase-deficient individuals (McSweeney *et al.,* 2004). There are also some promising data relating to the prevention and treatment of allergies and inflammatory bowel diseases. The proper enumeration of LAB species is crucial in studying of their role and their dynamics in different niches (Duthoit *et al.*, 2005a; Duthoit *et al.*, 2005b). In spite of the development of various selective culture media, LAB identification and enumeration in dairy products may cause some drawbacks (Dasen *et al.*, 2003). The fast and reliable identification and quantification of LAB species in complex food matrix such as cheese is possible thanks to the application of molecular biology techniques especially when the target population is very low and accounts for at least 1% of the total bacterial population (Amann *et al.*, 1995; Ben Amor *et al.*, 2007; Bouton *et al.*, 2002; Coppola *et al.*, 2001; Coppola *et al.*, 2006; Dahllof 2002). When target population is lower than 1%, PCR techniques are recommended for detection (De Candia *et al.*, 2007).

In the present paper, real-time quantitative PCR protocol was developed to specifically detect and quantify the LAB species comprising *Lactobacillus delbrueckii* and *Streptococcus thermophilus* in artisanal cheese produced from unpasteurised cow milk. The results of the study of molecular quantification were similar to those achieved by application of the classic plate count method.

Experimental

Materials and Methods

Extraction of DNA from pure cultures. *L. delbrueckii* CNRZ69, CNRZ207T, CNRZ334 and CNRZ1105 and *S. thermophilus* CNRZ440 and CNRZ1580 were propagated in 10 ml MRS and M17 broths, respectively, at 42°C. The bacteria coming from a 2-ml late exponential growth phase culture ($A_{650\ nm} = 0.7 - 0.8$) were collected by centrifugation at 3 000 × g for 5 min at 4–6°C and stored at –20°C until DNA extraction. One millilitre of enrichment culture was pipetted into a 2 ml microcentrifuge screw-cap tube and was centrifuged at 13 000 × g for 5 min. Then the supernatant was discarded using a pipet. The care was taken not to disrupt the pellet. Then 200 µl of Fast Lysis Buffer (Syngen Biotech, Germany) was added to the bacterial pellet, the tube was tightly capped and the pellet was resuspended by vigorous vortexing. The microcentrifuge tube was placed into a thermal shaker (800 × g) set to 100°C. The sample was heated for 10 min. The sample was removed and cooled to room temperature (15–25°C) for 2 min. The tube was centrifuged at 13 000 × g for 5 min. After centrifugation, the supernatant was carefully transferred to a new tube and 2 µl of this supernatant was used as the template.

Extraction of bacterial genomic DNA from cheese. DNA was isolated from cheese by using Syngen Food DNA Mini Kit (Syngen Biotech, Germany) according to manufacturer protocol. Two hundred mg of the homogenised commercial cheese was placed in 2 ml tube, then 1 ml of buffer DLF was added. The tube was closed and mixed by vortexing. The total volume of supernatant cannot be less than 700 µl. Thirty microlitres of proteinase K was added, the tube was closed and mixed by vortexing, then incubated at 60°C for 30 minutes. During the incubation, the sample was vortexed twice. The sample was incubated for 5 minutes on ice. Then it was centrifuged for 5 minutes at 2 500 × g. Seven hundred microlitres of the supernatant was transferred to a new 2 ml tube. In some food samples the three phases can be formed. In this case, 700 µl of the middle phase was transferred to a new 2 ml tube. Then 500 µl of chloroform was added, the tube was closed and vortexed for 15 seconds. Then the tube was centrifuged for 15 minutes at 14 000 × g. Then 350 µl of the upper phase was transferred to a new 2 ml tube, then 350 µl of buffer DWF was added. The lid was closed, the tube was vortexed for 10 seconds then centrifuged. The column DF was placed in a 2 ml tube. All the material was transferred into the column DF. The lid was closed. The tube was centrifuged for 30 seconds at 11 000 × g. The supernatant was discarded, and the column was transferred back to the tube. Seven hundred microlitres of buffer DPF was added to the column, then the lid of the column was closed. The column was centrifuged for 30 seconds at 18 000 × g. The supernatant was discarded, and the column was transferred back to the tube. The column was centrifuged for 3 minutes at 18 000 × g. The column was transferred to a new 1.5 ml tube. From one hundred to two hundred microlitres of pre-warmed DE elution buffer was added at the center of the membrane and incubated at room temperature for 1 minute. The lid was closed and the tube was centrifuged for 1 minute at maximum speed (18 000 × g).

Enumeration of bacteria and identification of LAB isolates. Serial dilutions of pure cultures and cheese sample were prepared in sterile 1% (wt/vol) peptone solution and plated on MRS agar medium with a spiral plating device (Eddy Jet, IUL, Barcelona, Spain). The acidified MRS agar medium (pH 5.5) was also applied to count a specific number of lactobacilli. Counting was

based on the morphology of colonies associated with the morphology of bacterial cells observed by microscopy. The species present in the cheese samples could easily be distinguished based on their respective morphologies. Isolates of representative lactobacilli were the subject of phenotypic characterization to confirm their classification. The facultatively heterofermentative species *L. rhamnosus*, *L. casei* and *L. paracasei*, produced gas when grown in MRS broth containing 4% sodium gluconate as the carbon source (facultative heterofermentation), whereas no gas was produced from glucose. *L. rhamnosus* strains were able to grow in MRS broth containing 0.5% glucose, galactose, mannitol or rhamnose as carbon sources, and could also grow at 45 and 48°C. *L. paracasei* and *L. casei* strains did not use rhamnose as a carbon source and could not grow at 48°C. *Lactobaccilus acidophilus* strains did not use gluconate, mannitol or rhamnose as carbon sources, while *L. delbrueckii* subsp. *bulgaricus* strains did not use galactose or the three carbon sources cited above.

Designing of PCR primers. Specific primers for the detection of *L. delbrueckii* and *S. thermophilus* were designed using an alignment of LAB 16S rRNA gene sequences obtained with the GeneBase software (Applied-Maths, St-Martens-Latem, Belgium). The sequences of the 16S rRNA genes were provided by GenBank (www.ncbi.nlm.nih.gov/Genbank/; Accession Number X52654 for *L. delbrueckii* and Accession Number X68418 for *S. thermophilus*). Sequences unique to *L. delbrueckii* and *S. thermophilus* were compared with those of closely related strains. The primer sets were designed using Primer Express Software v 3.0 (Applied Biosystems, Foster City, CA, USA). The sets were validated using NCBI BLAST (Basic Local Alignment Search Tool: www.ncbi.nlm.nih.gov/blast/). The sequences for *L. delbrueckii* were as follows: forward primer 5'-ACATGAATCGCATGATTCAAG-3'; reverse primer 5'-AACTCGGCTACGCATCATTG-3'. The sequences for *S. thermophilus* were as follows: forward primer 5'-TTATTTGAAAGGGGCAATTGCT-3'; reverse primer 5'-GTGAACTTTCCACTCTCACAC-3'. The oligonucleotides were synthesised and purchased from Eurofins Genomics (Germany).Fluorescence was detected using an optical detection system installed in the thermocycler of Stratagene Mx3005P (Real-Time PCR Detection System, Agilent Technologies, USA). Fluorescence data were collected during the annealing/elongation step of each PCR cycle. The software automatically plots the relative fluorescence unit (RFU) versus the PCR cycle number. The threshold cycle (Ct), which expresses the amount of a particular nucleic acid sample, is the number of amplification cycles needed for the accumulated fluorescence to achieve a value essentially higher than the background. A Ct value which exceeds 40 meant a negative result.

PCR fragments of 16s rRNA gene, standard curves for molecular quantification. The 16S rRNA gene fragments of *L. delbrueckii* and *S. thermophilus* were synthesised and purchased from Eurofins Genomics (Germany). They were delivered in the lyophilized form. For *L. delbrueckii* there were 1.43×10^{12} DNA copies. They were dissolved in 1430 µl of DE buffer (Syngen Biotech, Germany) achieving the concentration of 1×10^{9} DNA copies/µl of eluate. This concentration was used for preparation of standards for standard curve. The dilutions were prepared to achieve 1×10^{1} DNA copies/µl of eluate in the highest dilution.

For *S. thermophilus* there were also $1{,}43 \times 10^{12}$ DNA copies. They were dissolved in 1430 µl of DE buffer (Syngen Biotech, Germany) achieving the concentration of 1×10^{9} DNA copies/µl. This concentration was used for preparation of standards for standard curve. The dilutions were prepared to achieve 1×10^{1} DNA copies/µl of eluate in the highest dilution.

A 10-fold dilution series of the PCR fragment solution for each bacterial species, covering 7 logs ranging from 10^{1} to 10^{7} DNA copies per reaction, was used to estimate the sensitivity of the method. The correlation coefficients (r^2) of the standard curves for both *L. delbrueckii* and *S. thermophilus* were equal to 0.999 for the initial copy numbers of standard PCR fragments within the range of 10^{1}–10^{7} per reaction, indicating that the exponential amplification patterns were reliable under these conditions. All assays were performed at least in triplicate and the average values were used for analysis.

Construction of standard curves. Standard curves were prepared with serial dilutions of genomic DNA isolated from *L. delbrueckii* and *S. thermophilus* and cheese. Accession Number X52654 for *L. delbrueckii* and Accession Number X68418 for *S. thermophilus*). The number of bacterial DNA copies were calculated on the basis of the size of the *L. delbrueckii* (GenBank accession number X52654) and *S. thermophilus* (GenBank accession number X68418) using Avogadro's constant (6.023×10^{23}) and the molecular weight of DNA (660 Da/bp). Genomic DNA was tenfold serially diluted in ultrapure water to final concentrations ranging from 10^{7} to 10^{1} genome copies per 1 µl, equivalent to concentrations of 33.2 to 3.32×10^{-6} ng. The Cq versus log CFU was estimated using genomic DNA extracted from the bacteria culture grown until stationary growth phase (OD 0.8) or from cheese with *L. delbrueckii* and *S. thermophilus*. Ten times serial dilutions of DNA extracted from *L. delbrueckii* and *S. thermophilus* was performed and the corresponding CFU was calculated based on plate counting of the same sample, bacterial culture or cheese with *L. delbrueckii* and *S. thermophilus*. Standard curves were generated by the plot cycle threshold (Cq) values versus logarithm of bacterial DNA copy number (pure culture) or CFU (pure culture and cheese). Amplification

efficiencies were determined using the following equation: $E = 10^{(-1/S)} - 1$; where E is the efficiency and s is the slope obtained from the standard curve.

Relationship between colony forming units and number of target copies. The number of *L. delbrueckii* was 7.3×10^6 CFU/ml. In comparison, 1.2×10^7 copies/ml were achieved in the qPCR assay. Based on these results, a ratio of 1.6 copies per CFU was calculated for *L. delbrueckii* (1.2×10^7 copies/ml amounted to 7.3×10^6 CFU/ml). Similar ratios were achieved for *S. thermophilus*. (1.9 copies per CFU); The enumeration of the liquid stock culture of *S. thermophilus* amounted to 6.9×10^6 CFU/ml. In comparison, 1.3×10^7 copies/ml were achieved in the qPCR assay. Based on these results, a ratio of 1.9 copies per CFU was calculated for *S. thermophilus* (1.3×10^7 copies/ml amounted to 6.9×10^6 CFU/ml) (Table I). Since cells of LAB occur sometimes in pairs, it is plausible that the CFU counts are usually lower than the number of target copies obtained in the qPCR assay.

Real-time PCR conditions. The reaction total volume was 20 µl. Real-time PCR analysis was performed using the thermocycler of Stratagene Mx3005P (Agilent Technologies, USA). The PCR mixture contained 5 µl DNA template, 4 µl of Quantum EvaGreen PCR Mix (Syngen Biotech, United Kingdom), 0.5 µl of primers F and R respectively, and 10 µl of PCR water. A non-template control (NTC) contained 5 µl of water instead of DNA and was included in each run. The real-time PCR cycling parameters were the following: 1 cycle of amplification (95°C for 5 min) and 35 cycles of amplification (94°C for 30 s, 60°C for 30 s, 72°C for 90 s). The real-time PCR reaction and amplification step were carried out using a DNA amplification curves which were the subject of analysis. The calculation of the threshold cycle (C_T) value was carried out using Stratagene Mx3005P software version 2.1 (Agilent Technologies, USA). The C_T value was described as the real-time PCR cycle, at which the generated fluorescence increased exponentially and exceeded its background level.

Table I
Counts of *L. delbrueckii* and *S. thermophilus* in stock culture.

Strain	Quantification in stock culture with PCM* (CFU/ml)	Quantification in stock culture with qPCR (copies/ml)	Number of DNA copies per CFU
L. delbrueckii	7.3×10^6	1.2×10^7	1.6
S. thermophilus	6.9×10^6	1.3×10^7	1.9

* PCM, plate count method.

Statistical analysis. Each experiment was repeated at least three times and data were analysed using analysis of variance (ANOVA) and Duncan multiple range test ($P < 0.05$).

Results

Assessment of the real-time PCR assay performance. The qPCR-standards (linearised plasmids containing the target sequences) for *L. delbrueckii* and *S. thermophilus* species were tenfold serial diluted to achieve a concentration in the range from 10^7 to 10^1 DNA copies per reaction. For both primer-systems, the result of the qPCR indicated a linear correlation between the concentrations of the qPCR-standards and the threshold cycles (Ct) over a 7-log range (Fig. 1

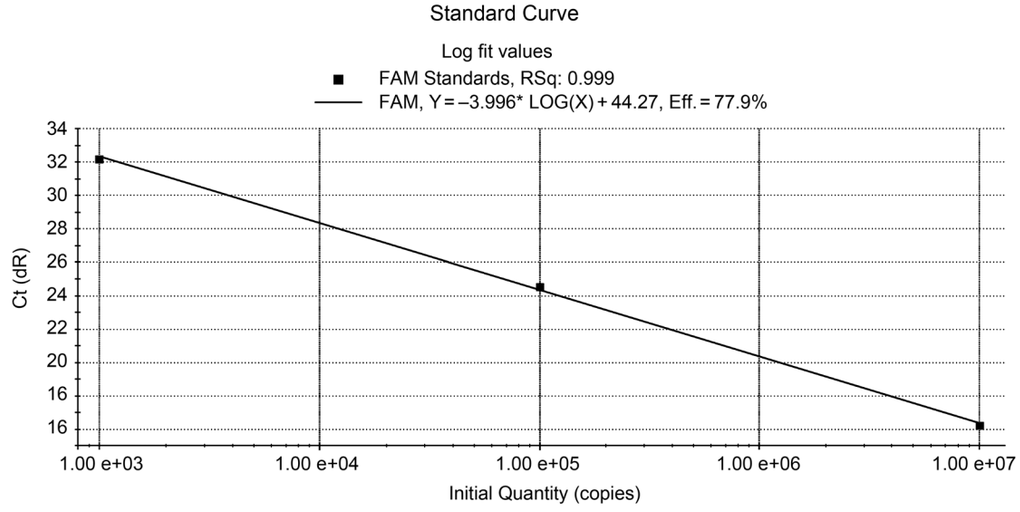

Fig. 1. Performance of the qPCR assays for the species-specific quantification of *L. delbrueckii*. The standard curve was made by plotting the inputs of the three different concentrations of qPCR standard against Ct outcomes. Each data point constitutes a mean value and standard deviation of three qPCR replicates.

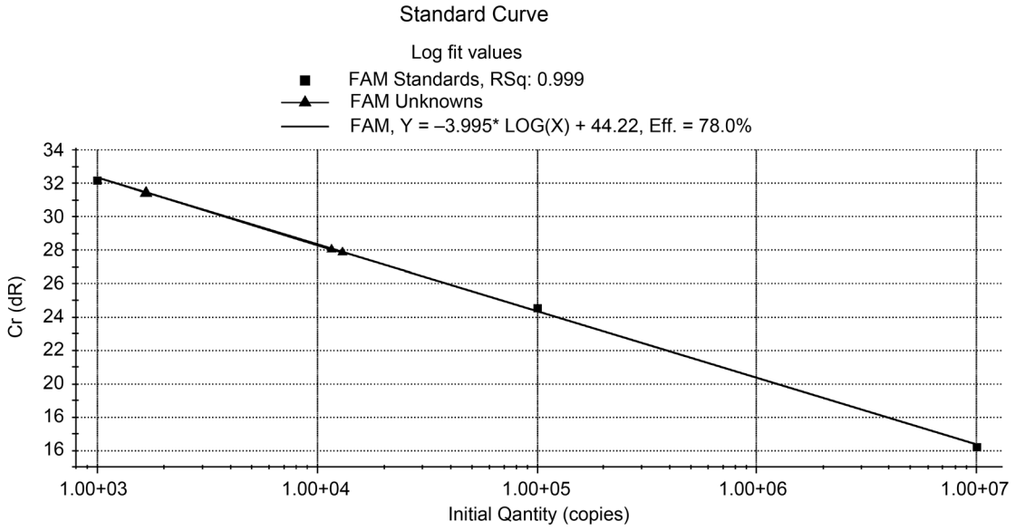

Fig. 2. Performance of the qPCR assays for the species-specific quantification of *L. delbrueckii*. The standard curve was made by plotting the inputs of the four different concentrations of qPCR standard against Ct outcomes. Each data point constitutes a mean value and standard deviation of three qPCR replicates.

and Fig. 3). The coefficients of correlation (R^2) over the whole range were 0.999 for *L. delbrueckii* and *S. thermophilus*. The amplification efficiency (Eff.) was 77.9% for *L. delbrueckii* and 93.5% for *S. thermophilus*.

Assessment of primer specificity for *L. delbrueckii* and *S. thermophilus*. Specificity test was carried out using DNA extracted from *L. delbrueckii* and *S. thermophilus* strains and other bacterial genera to evaluate if the primer pairs with the sequences described in Materials and Methods were able to detect exclusively *L. delbrueckii* and *S. thermophilus*. Specificity test was carried out using bacterial DNA extracted from pure medium cultures and from cheese produced from unpasteurised milk (10 ng of template DNA). *L. delbrueckii* strains CNRZ69, CNRZ207T, CNRZ334 and CNRZ1105 presented mean Ct values ±standard deviation equal to 16.2 ± 0.7, 17.2 ± 0.4, 18.2 ± 0.2 and 18.7 ± 0.2 respectively, and amplicon presented Tm of 78.3 ± 0.2, 78.0 ± 0.2, 77.9 ± 0.2 and 77.3 ± 0.2 respectively (Table II).

S. thermophilus strains CNRZ440 and CNRZ1580 presented mean Ct values ± standard deviation equal to 17.2 ± 0.4 and 18.2 ± 0.4 respectively, and amplicon presented Tm of 78.8 ± 0.2 and 78.2 ± 0.2 respectively (Table II). *Bifidobacterium* BB-12 and *Lactobacillus plantarum* did not show amplification as expected for all qPCR assays. Other negative samples indicated some unspecific amplification, with late Ct and different Tm values in comparison to *L. delbrueckii* and *S. thermophilus* Tm values in a range from 77.3 to 78.8. *Bacillus cereus* indicated Ct = 31.7. *Escherichia coli*, *Pseudomonas* spp., and *L. acidophilus* also showed unspecific amplification at late Ct values (Table II). *L. acidophilus* indicated a late Ct (Ct > 39.42). *E. coli* and *Pseudomonas* presented late Ct (Ct > 37.89 and Ct > 38.73, respectively) with different Tm values.

qPCR parameters for enumeration of *L. delbrueckii* and *S. thermophilus*. The reaction parameters such as efficiency and correlation coefficient of the qPCR assay with the application of specific primer pairs were determined based on standard curves achieved from tenfold serial dilution of the qPCR-standards (linearised plasmids containing the target sequences of bacterial DNA) for *L. delbrueckii* (Fig. 1) and *S. thermophilus* species (Fig. 3). The standard curves indicated the

Table II
Ct and Tm achieved by qPCR assay using DNA extracted from *L. delbrueckii* and *S. thermophilus* (positive controls) and other bacterial species (negative controls).

Sample*	Ct	Tm
L. delbrueckii CNRZ69	16.2 ± 0.7	78.3 ± 0.2
L. delbrueckii CNRZ207T	17.2 ± 0.4	78.0 ± 0.2
L. delbrueckii CNRZ334	18.2 ±0.2	77.9 ±0.2
L. delbrueckii CNRZ1105	18.7 ± 0.2	77.3 ± 0.2
S. thermophilus CNRZ440	17.2 ± 0.4	78.8 ± 0.2
S. thermophilus CNRZ1580	18.2 ± 0.4	78.2 ± 0.2
Bifidobacterium BB-12	nd	nd
L. plantarum	nd	nd
B. cereus	34.7	78.2 ± 0.2
E. coli	37.89	65.2 ± 0.2
Pseudomonas spp.	38.73	75.2 ± 0.2
Lactobacillus acidophilus	39.42	72.2 ± 0.2

* 10 ng of template DNA; nd; not detected with Ct > 40

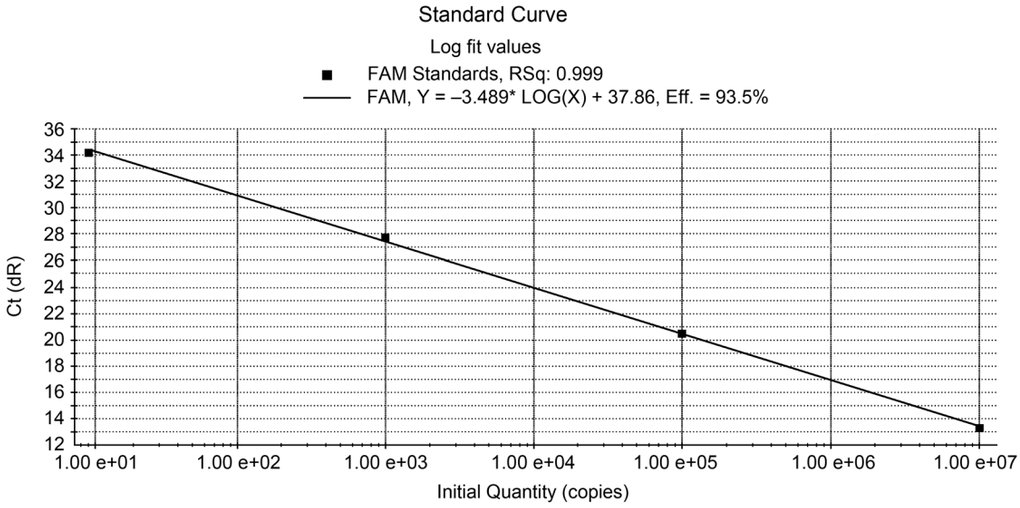

Fig. 3. Performance of qPCR assays for the species-specific quantification of *S. thermophilus* in cheese (▲). The standard curve was made by plotting the inputs of the three different concentrations of qPCR standard (■) against Ct outcomes. Each data point constitutes a mean value and standard deviation of three qPCR replicates.

suitable linear correlation coefficient $R^2 = 0.999$ and mean efficiency Eff = 77.9% for *L. delbrueckii* and the suitable linear correlation coefficient $R^2 = 0.999$ and mean efficiency Eff = 93.5% for *S. thermophilus*. The limit of detection (LOD) for *L. delbrueckii* was 3 log DNA copy number, corresponding to 3.32 pg of DNA (Fig. 1). For *L. delbrueckii* LOD corresponded to a mean Ct = 32.0. The Ct versus log CFU of *L. delbrueckii* was estimated using genomic DNA extracted from the *L. delbrueckii* bacterial culture plate counted in parallel, so tenfold serial dilutions of bacterial DNA were performed and the corresponding CFU values were calculated based on plate counting. LOD corresponded to 2.78 log CFU of *L. delbrueckii*.

The qPCR enumeration of *L. delbrueckii* in cheese samples was carried out on the base of the construction of standard curve achieved from tenfold serial dilution of the qPCR-standards (linearised plasmids containing the target sequences of bacterial DNA) for *L. delbrueckii* and bacterial DNA coming from *L. delbrueckii* present in cheese. In this case, the efficiency value was 78.0% and R^2 was 0.99 (Fig. 2). The number of DNA copies in the cheese samples taken from the three different places amounted to 1.68×10^3/µl; $1{,}17 \times 10^4$/µl; 1.30×10^4/µl of eluate per reaction (Fig. 2). The standard curve equation Ct versus log CFU of samples of cheese for *L. delbrueckii* (Fig. 2) was used to calculate CFU per reaction well of all cheese samples from obtained Ct values. It was possible to obtain *L. delbrueckii* count (CFU/g of cheese) of cheese samples by qPCR taking into consideration the fact that 1.6 DNA copies present in *L. delbrueckii* corresponds to 1 CFU (Table I and Table III). On the other hand, 1.9 DNA copies present in *S. thermophilus* corresponds to 1 CFU (Table I and Table III).

Table III
Comparison of *L. delbrueckii* and *S. thermophilus* count (log CFU/g) in cheese obtained by qPCR and plate count method.

Samples	CFU/g with plate count method	CFU/g with qPCR method	DNA copies with qPCR method
L. delbrueckii	1.02×10^3 7.23×10^3 8.05×10^3	1.05×10^3 7.31×10^3 8.12×10^3	1.68×10^3 1.17×10^4 1.30×10^4
S. thermophilus	5.42×10^4 8.65×10^4 2.02×10^5	5.52×10^4 8.74×10^4 2.09×10^5	1.05×10^5 1.66×10^5 3.98×10^5

The numbers of DNA copies coming from *S. thermophilus* present in the cheese samples are present in Figure 4.

Cheese samples were analysed with plate count method and qPCR in parallel. Compared with the classic plate count method, the newly developed qPCR method gave faster and species specific determination of two dairy LAB and yielded comparable quantitative results.

Discussion

The bacterial quantification in food with the application of a DNA-based method requires the proper DNA extraction method. It is very difficult to extract bacterial DNA, especially from a dairy product, as it may potentially contain PCR inhibitors like calcium and fat. In the present study, Syngen Food DNA Mini Kit protocol was used to extract DNA from commercial cheese produced from unpasteurised milk. Such DNA

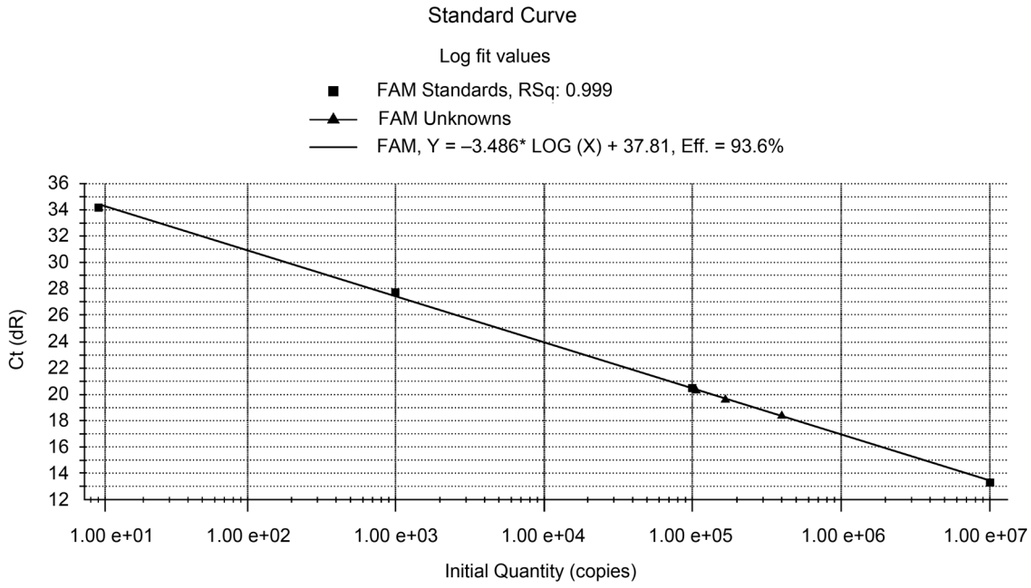

Fig. 4. Performance of the qPCR assays for the species-specific quantification of *S. thermophilus* in cheese (▲). The standard curve was made by plotting the inputs of the four different concentrations of qPCR standard (■) against Ct outcomes. Each data point constitutes a mean value and standard deviation of three qPCR replicates.

was used for qPCR enumeration assay of *L. delbrueckii* and *S. thermophilus* in cheese. A qPCR assay based on 16S rRNA gene sequences was successfully applied for enumeration of *L. delbrueckii* and *S. thermophilus* in cheese (Jany and Barbier, 2008; Randazzo *et al.*, 2002). Several other lactic acid bacteria were used as negative controls by other authors, and the pairs of primers used in this qPCR assay were specific enough for the identification of *L. delbrueckii* and *S. thermophilus* (Justé *et al.,* 2008). The specificity of these primers was also examined with other bacterial species (Table II).

It was found that some bacterial species showed unspecific amplification; however, Ct values were always above the Ct value corresponding to LOD for *L. delbrueckii* (Ct = 32) and LOD for *S. thermophilus* (Ct = 34), and they showed amplicons with different Tm values in comparison to compared to *L. delbrueckii* and *S. thermophilus* samples (Table II). The ΔCt between DNA samples (10 ng) of *L. delbrueckii* and *S. thermophilus* strains (Cq < 18) and the other bacteria (Cq < 34) is sufficiently high to reinforce the use of the specific primer pairs used in this study because the amplifications of other bacterial DNA of *Bifidobacterium* BB-12, *L. plantarum*, *B. cereus*, *E. coli*, *Pseudomonas* spp. and *L. acidophilus* are unspecific and they are easily distinguishable by their Tm. The DNA extraction method possesses a huge impact on the quantification by real-time PCR and it is essential to achieve an optimal yield of DNA and to avoid the appearance of substances that might influence PCR efficiency (Mannu *et al.,* 2002; Sánchez *et al.*, 2006). The standard curve for *L. delbrueckii* showed a relatively high efficiency value of 77.9% and a very high efficiency value of 93.5% for *S. thermophilus*.

It should be noted that the qPCR assay designed for one matrix may not occur to be proper for other matrices. Limit of detection (LOD) which is defined as the lowest amount of sample that can be reliably detected amounts to 3 log DNA copy number for *L. delbrueckii* and 1 log DNA copy number for *S. thermophilus*. This *L. delbrueckii* qPCR assay enabled to detect *L. delbrueckii* DNA ranging between 7 log genome copies to 3 log genome copies in the reaction well in comparison to *S. thermophilus* qPCR assay which enabled to detect *S. thermophilus* DNA ranging between 7 log genome copies to 1 log genome copy in the reaction well (Ventura *et al.*, 2003). It is a proper amount of LAB in cheese samples, once they should be contained in relatively high numbers.

L. delbrueckii species were presented in the range 1.02×10^3–8.05×10^3 log CFU/ml using the plate count method and in the range 1.05×10^3–8.12×10^3 log CFU/ml using qPCR method. *S. thermophilus* species were presented in the range 5.42×10^4–2.02×10^5 log CFU/ml using the plate count method and in the range 5.52×10^4–2.09×10^5 log CFU/ml using qPCR method. It was observed that both plate count method and qPCR method gave very similar results concerning a number of *L. delbrueckii* and *S. thermophilus* in cheese samples. However, in comparison to the classic plate count method, the newly developed qPCR method gave faster and species specific determination of two dairy LAB (Giraffa and Neviani, 2000).

The results of the present work constitute a molecular approach to identify the characteristic microflora of traditional Polish artisanal cheese. Further isolation of the LAB strains together with further examination and enumeration of other specific species could provide us with a wider knowledge of bacterial ecosystem of this traditional cheese.

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ORIGINAL PAPER

The Determination and Correlation of Various Virulence Genes, ESBL, Serum Bactericidal Effect and Biofilm Formation of Clinical Isolated Classical *Klebsiella pneumoniae* and Hypervirulent *Klebsiella pneumoniae* from Respiratory Tract Infected Patients

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Abstract

Klebsiella pneumoniae strains that are commonly recognized by clinicians and microbiologists are termed as classical *K. pneumoniae* (c*KP*). A strain with capsule-associated mucopolysaccharide web is known as hypervirulent *K. pneumoniae* (hv*KP*) as it enhances the serum resistant and biofilm production. Aim is to determine and correlate various virulence genes, ESBL, serum bactericidal effect and biofilm formation of clinical isolated c*KP* and hv*KP* from respiratory tract infected patients. A total of 96 *K. pneumoniae* strains were isolated from sputum of respiratory tract infected patients. The isolates were performed string test, AST, ESBL virulence gene, serum bactericidal and biofilm assays. Out of 96 isolates, 39 isolates (40.6%) were identified with hypervirulent phenotypes. The number of c*KP* exhibiting resistance to the tested antimicrobials and ESBLs were significantly higher than that of the hv*KP* strains. The virulence genes of *K. pneumoniae* such as K1, K2, *rmp*A, *uge*, *kfu* and aerobactin were strongly associated with hv*KP* than c*KP*. However, no significant difference was found in FIM-1 and MrKD3 genes. ESBL producing c*KP* and hv*KP* were significantly associated with strong biofilm formation (both $P < 0.05$) and highly associated with bactericidal effect of serum (both $P < 0.05$) than c*KP* strains. However, neither biofilm formation nor bactericidal effect of serum was found with significant difference in between ESBL producing c*KP* and ESBL producing hv*KP* strains (both $P > 0.05$). Although the hv*KP* possess more virulence gene, but they didn't show any significant difference between biofilm formation and bactericidal effect of serum compared with ESBL producing c*KP* strains.

Key words: biofilm, ESBL, classical *K. pneumoniae vs* hypervirulent *K. pneumoniae*, serum resistance, virulence genes

Introduction

Klebsiella pneumoniae is an opportunistic pathogen of the *Enterobacteriaceae* family and principally causes pneumonia (Podschun and Ullmann, 1998), and also is associated with pyogenic liver abscesses over the past decade (Wang *et al*., 1998) and it has been implicated in 7–12% of hospital-acquired pneumonia in ICUs in the United States (1997). It typically expresses different virulence factor genes such as a smooth lipopolysaccharide (O antigen) and capsule polysaccharide (K antigen) on its surface (Kenne *et al*., 1983). There are at least 77 capsular serotypes defined, and serotype-related variation in the infection severity has been observed. Out of 77 capsular serotypes (K), the strains with capsular serotypes K1 and K2 have been identified as the predominant virulent strains, and their virulence has been confirmed in mouse models (Fung *et al*., 2002). The *rmp*A is a transcriptional activator of capsular polysaccharide (CPS) gene transcription, CPS synthesis and HV in *K. pneumoniae* K1/K2 (Lai *et al*., 2003). The *uge* gene encodes uridine diphosphate galacturonate 4-epimerase which expresses both smooth lipopolysaccharide with O antigen molecules and CPS with K antigen on the surface. Aerobactin is a siderophore that aids the bacterium in its competition with the host for iron (Quinn, 1994). The *kfu* gene encodes for an iron uptake system which is a significantly associated with the purulent tissue infections and HV phenotype (Ma *et al*., 2005). The *fim*H (or *fim*H-1) and *mrk*D genes which are relevant to type 1 and type 3 fimbriae respectively are responsible for attachment to host cells (Podschun and Ullmann, 1998). These all factors contribute to virulence and are important for colonization, invasion and pathogenicity.

K. pneumoniae strains usually recognized by microbiologists and clinicians are termed as c*KP*. Such strains are scandalous for their capability to cause acquired

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hospital infections and acquire multidrug resistant especially extended-spectrum beta lactamase (ESBL) that has led the treatment to limited options (Ko *et al*., 2002; Podschun and Ullmann, 1998). ESBLs are plasmid mediated enzymes and it inactivate β-lactam antibiotics such as oxyimino-cephalosporins and oxyimino-monobactam, except cephamycins and carbapenems (Paterson and Bonomo, 2005) and it is inhibited by clavulanic acid and placed it under Bush's functional class 2be (Bush *et al*., 1995). Till the date more than 200 different types of ESBLs have been found. There is a increase in the prevalence of TEM, SHV and CTX-M type of ESBLs among the *Enterobacteriaceae* in Europe and Asia (Bonnet, 2004). A new variant of *K. pneumoniae*, designated as HV *K. pneumoniae* due to the high production of mucopolysaccharide was first described in 1986 by a Taiwanese doctors. The HV phenotype is also known as hv*KP*, and it enhances the biofilm production and resistance to serum bactericidal activity. Biofilm is a complex polymer matrix composed of cells and matrix materials. The serum bactericidal activity is mediated by the complement proteins through the complement pathway. The complement pathways lead, *via* the activation of C3, for the formation of the opsonin C3b, which finally results in the formation of the terminal C5b-C9 complex and thus plays a key role in this defense system (Tomas *et al*., 1986). Many studies identified that hv*KP* strains produced more biofilm and are less susceptible to human serum than c*KP* strains (Li *et al*., 2014; Wu *et al*., 2011). Some studies have addressed that ESBL producing strain formed heavy biofilm than non-ESBL producing strains of *K. pneumoniae* (Yang and Zhang, 2008) and recently another study have been shown that the serum-resistant strains are significantly more general among ESBL-producing *K. pneumoniae* strains than among non-ESBL producers (Sahly *et al*., 2004). In view of previous findings, the goal of this study is to identify and correlate the various virulence genes, biofilm formation, bactericidal effect of serum and ESBL in between hv*KP* strain and c*KP* strain. To the best of our knowledge, the combine study of virulence genes, biofilm formation, bactericidal effect of serum and ESBL in between hv*KP* strain and c*KP* strain have not been previously reported.

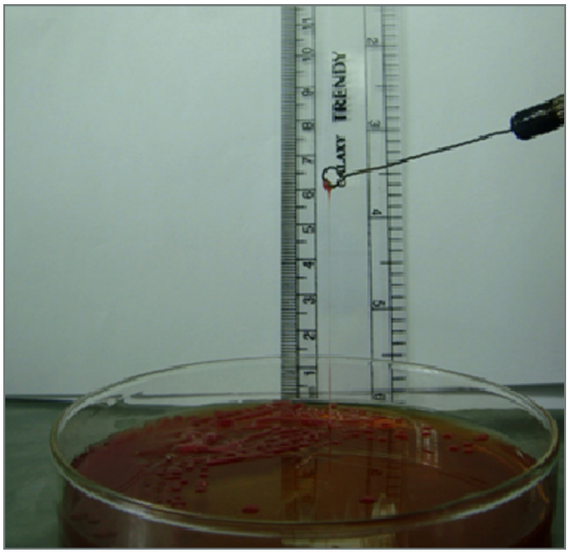

Fig. 1. Positive string test (Mucoviscous string > 5 mm on Agar plate).

Experimental

Materials and Methods

Bacterial strains and HV testing. A total of 96 *K. pneumoniae* were isolated from sputum of pneumonic patients and were collected from a period of March 2013 to October 2014 in Shenyang Hospital of Liaoning Province and Tongliao Hospital of Nei Menggu province in China. *K. pneumoniae* strains were isolated from sputum that were identified and confirmed by standard methods (Farmer, 2003).

HV testing was done by string test. The string test was performed to distinguish hv*KP* from c*KP* strains. The string test was defined as the positive when the formation of a mucoviscous string of > 5 mm was observed, by using a bacteriology inoculation loop to stretch a colony that was grown overnight on an agar plate at 37°C. A positive string test with *K. pneumoniae* strains were designated as hv*KP* in Fig. 1 (Fang *et al*., 2004).

Antimicrobial susceptibility testing and ESBLs detection. Susceptibility testing for the 96 *K. pneumoniae* strains was carried out with disc diffusion methods. The control strains were used as *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, and *Escherichia coli* ATCC 25922. The interpretation of results were recorded according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2015). Antimicrobial agents tested included ampicillin, piperacillin, cefazolin, cefuroxime, cefoxitine, cefoperazon, ceftriaxone, cefotaxime, ceftazidime, cefepime, meropenem, imipenem, aztreonam, amikacin, gentamicin, trimethoprim-sulfamethoxazole, and ciprofloxacin. These all antibiotics were purchased from Oxoid company, UK.

The combination-disk synergy tests using ceftazidime (30 μg) ± clavulanic acid (10 μg) and cefotaxime (30 μg) ± clavulanic acid (10 μg) were performed to detect the phenotype of ESBLs for all the collected isolates. The phenotype of ESBLs was confirmed by 5 mm or more increased zone diameter for the combination of clavulanic acid with either cefotaxime or ceftazidime versus its zone when tested alone. The ESBL negative

and positive strains were used as *E. coli* (ATCC 25922) and *K. pneumoniae* (ATCC 700603) respectively.

PCR for amplification of ESBL and virulence associated genes. The template DNA was prepared from bacterial colonies. The colonies of bacteria were picked and suspended in 100 µl of mili-Q water. The suspensions of sample were boiled for 15 min and rapid cool at –20°C for 5 min. The bacterial cell debris was separated by centrifugation for 10 min at 15,000 rpm and the supernatant was collected and used as a template DNA. The DNA concentration was measured by using Epoch Gen5 CHS 2.01. The PCR specific primers and cycling condition used to detect the target gene are shown in Table I and II respectively. The PCR product from each of the detected genes was sequenced and compared with *K. pneumonie* DNA sequences on BLAST of NCBI (http://blast.ncbi.nlm.nih.gov/). Each of the target gene sequenced showed a high level of identification (> 98% homology) with the published target sequence (Gen Bank accession number: KF77780.1 for *mrk*D; FJ483592.1 for *fim*H-1; AB355924.1 for *mag*A (K1); AB362367.1 for *wzy* (K2); X17518.1 for

Table I
Primers used to detect the target gene sequences.

Target gene	Sequences of Primer (5'-3')	Size of amplified product (bp)
FimH-1	F: ATG AAC GCC TGG TCC TTT GC R: GCT GAA CGC CTA TCC CCT GC	**688**
*mrk*D	F: CCA CCA ACT ATT CCC TCG AA R: ATG GAA CCC ACA TCG ACA TT	240
*mag*A (K1)	F: GGT GCT CTT TAC ATC ATT GC R: GCA ATG GCC ATT TGC GTT AG	**1 282**
wzy (K2)	F: GAC CCG ATA TTC ATA CTT GAC AGA G R: CCT GAA GTA AAA TCG TAA ATA GAT GGC	**641**
*rmp*A	F: ACT GGG CTA CCT CTG CTT CA R: CTT GCA TGA GCC ATC TTT CA	**535**
uge	F: TCT TCA CGC CTT CCT TCA CT R: GAT CAT CCG GTC TCC CTG TA	**534**
kfu	F: GAA GTG ACG CTG TTT CTG GC R: TTT CGT GTG GCC AGT GAC TC	**797**
aerobactin	F: GCA TAG GCG GAT ACG AAC AT R: CAC AGG GCA ATT GCT TAC C T	**556**
TEM	F: ATA AAA TTC TTG AAG ACG AAA R: GAC AGT TAC CAA TGC TTA ATC	**1 080**
SHV	F: GGG TTA TTC TTA TTT GTC GC R: TTA GCG TTG CCA GTG CTC	**930**
CTX-M	F: SCS ATG TGC AGY ACC AGT AA R: ACC AGA AYV AGC GGB GC	**585**

Table II
Cycling condition for PCR.

Target gene	Cycling Conditions					
FimH-1	95°C 4 min	95°C 1 min	57°C 1 min	72°C 1 min	72°C 10 min	Repeated for 35 cycles
*mrk*D	95°C 4 min	95°C 45 sec	55°C 45 sec	72°C 45 sec	72°C 7 min	Repeated for 30 cycles
*mag*A (K1)	95°C 4 min	95°C 45 sec	59°C 45 sec	72°C 2 min	72°C 9 min	Repeated for 30 cycles
wzy (K2)	95°C 4 min	95°C 45 sec	63°C 45 sec	72°C 1 min	72°C 9 min	Repeated for 35 cycles
*rmp*A	95°C 4 min	95°C 45 sec	52°C 45 sec	72°C 1 min	72°C 7 min	Repeated for 30 cycles
uge	95°C 4 min	95°C 45 sec	55°C 45 sec	72°C 1 min	72°C 7 min	Repeated for 30 cycles
kfu	95°C 3 min	95°C 45 sec	59°C 45 sec	72°C 1 min	72°C 7 min	Repeated for 35 cycles
aerobactin	95°C 5 min	95°C 1 min	54°C 1 min	72°C 1 min	72°C 7 min	Repeated for 35 cycles
SHV	95°C 4 min	95°C 45 sec	56°C 45 sec	72°C 1 min	72°C 7 min	Repeated for 35 cycles
TEM	94°C 4 min	94°C 45 sec	55°C 45 sec	72°C 1 min	72°C 7 min	Repeated for 35 cycles
CTX-M	95°C 4 min	95°C 45 sec	58°C 45 sec	72°C 1min	72°C 7 min	Repeated for 35 cycles

*rmp*A; 633804.1 for *uge*; KJ633800.1 for *kfu*; X98099.1 for SHV, HM131427.1 for TEM; HQ214044.1 for CTX-M). These genes were chosen as the positive control for the consequent PCR experiments.

Serum bactericidal assays. Normal human serum was obtained from the healthy adult volunteers. The sera were stored as aliquots at –70°C. *K. pneumonia* strains were determined by an established method (Podschun *et al*., 1993). An inoculum of 2.5×10^4 CFU, prepared from the mid-log phase, was mixed at a 1:3 vol/vol ratio with normal human serum. The final mixture, comprising 75% serum by volume, was incubated at 37°C for 3 hours, and 100 µl from each well was taken out for CFU determination before and after incubation at 37°C. The rate of survival was expressed as the number of viable bacteria treated with serum compared to the number of pretreatment. The assay was performed in triplicate and repeated three times.

Interpretation of results: following the criteria used by Benge (1988). The isolates were regarded as serum sensitive if at 3 h the viable counts were reduced to < 1% of the initial counts and resistant if > 90% of the organisms were still viable. Isolates having survival rates of 1–90% were regarded as intermediate sensitive.

Biofilm formation assays. Microtiter plate method was carried out according to Stepanovic *et al.* (2007) with a few modifications. Briefly, *K. pneumoniae* strains were grown overnight at 37°C in Mueller Hinton broth. The culture was adjusted to 0.5 McFarland then diluted 1:100 in the Mueller Hinton broth. Three wells of a sterile 48-well plastic tissue culture plate with a lid were filled with 1000 µl of diluted bacterial culture each. The negative control wells contained MH broth only. The plates were covered and incubated at 37°C for 24 h. The content of each well was aspirated, and was washed three times with normal saline (to remove free-floating "planktonic" bacteria). Biofilms formed by bacteria adherent "sessile" to the wells were heat-fixed by exposing them to hot air at 60°C for 3 hours and stained with 0.5% of Crystal Violet for 15 min. Excess stain was rinsed off with running tap water and the plates were dried. To quantify biofilm biomass, the crystal violet dye bound to the adherent cells was dissolved by adding 1000 µl of 33% acetic acid solution to each well and after 10 min, the OD of each well was measured at 595 nm. Each assay was performed in triplicate and repeated at least three times.

The interpretation of biofilm formation was done according to the criteria of Stepanovic *et al.* (2007).

Interpretation of biofilm formation results

Average OD value	Biofilm production
≤ ODc / ODc < ~ ≤ 2x ODc	Non/weak
2x ODc < ~ ≤ 4x ODc	Moderate
> 4x ODc	Strong

Note: ODc = average OD of negative control + 3x standard deviation of negative control optical density cut-off value (ODc)

Statistical analysis. The statistical analyses were performed using Statistical Package for Social Science 21.0. The descriptive data were reported as mean ± SD and percentage. The normally distribution of the data was performed by Kolmogorov-Smirnov test. For the differences in the outcomes between various groups, categorical variables were compared using chi-square analysis. When the number of cases was smaller than 5, the Fisher's exact test was used. The P value < 0.05 was defined as a significance.

Ethics statement. For collection of normal human serum from healthy adult volunteers and sputum from pneumonic patients, the method and the respective consent of documents were approved by the Ethics Committee of the Norman Bethune Health Science Center, Jilin University, China. The written informed consent is provided by all volunteers.

Results

Bacterial strains and HV of *K. pneumoniae* strains. The samples were collected from March 2013 to October 2014, and a total of 96 patients were diagnosed as suffering pneumonia with the culture-positive *K. pneumoniae*. Out of them, 71 (74.0%) were males and 25 (26.0%) were females. The mean age was 64.8 ± 15.4 years. Based on the results of the modified string test HV phenotypes were identified in 39 (40.6%) of the 96 isolates. The isolation of c*KP* and hv*KP* strains were obtained as 57 (59.4%) and 39 (40.6%), respectively. A significantly higher number of patients with c*KP* (P = 0.009) was detected. Neither age nor sex was associated with positive string test (both P > 0.05).

Antimicrobial resistance and ESBL. The number of c*KP* strains exhibiting resistance to the tested antimicrobials was significantly higher than that of the hv*KP* strains, with the exception of ampicillin, piperacillin, cefuroxime, cefoxitine, cefoperazone, imopenem, meropenem and ciprofloxacin, shown in Table III. The results of the combined disk test confirmed that 37 (38.5%) isolates were ESBL-producing strains. ESBL were identified in more number of c*KP* strains (28/57 [49.1%]) than in hv*KP* strains (11/39 [28.2%]) (P = 0.040). These results indicate a significant negative association between the ESBL producer and the HV phenotype in these isolates. In addition, single and multiple types of ESBL genes were present in c*KP* strains, whereas, only single gene was present in hv*KP* strains, shown in Table IV.

Virulence genetic characteristics of *K. pneumoniae*. The pneumonia is caused by *K. pneumoniae* strains

Table III
The percentage of antimicrobial resistance of *c*KP strain and *hv*KP strain.

	*c*KP (n = 57) No. (%)	*hv*KP (n = 39) No. (%)	P – value
Ampicillin	51 (89.5)	37 (94.9)	0.347
Pipracillin	27 (47.4)	14 (35.9)	0.264
Cefazolin	33 (57.9)	10 (25.6)	0.002*
Cefuroxime	31 (54.4)	15 (38.5)	0.125
Cefoxitin	14 (24.6)	6 (15.4)	0.277
Cefoperazon	12 (21.1)	4 (10.3)	0.163
Ceftriaxone	31 (54.4)	8 (20.5)	0.001*
Cefotaxime	28 (49.1)	9 (23.1)	0.010*
Ceftazidime	23 (40.4)	7 (17.9)	0.020*
Cefepime	24 (42.1)	2 (5.1)	0.001*
Aztreonam	25 (43.9)	4 (10.4)	0.001*
Imipenem	3 (5.3)	1 (2.6)	0.644
Meropenem	4 (7.0)	0 (0)	0.144
Amikacin	18 (31.6)	2 (5.1)	0.002*
Gentamycin	26 (45.6)	10 (25.6)	0.047*
Trimethoprim-sulfamethoxazole	28 (49.1)	8 (20.5)	0 .004*
Ciprofloxacin	15 (26.3)	9 (23.1)	0.719
ESBL total	28 (49.1%)	11 (28.2%)	0 .040*

* P < 0.05 is significant

that were encoded following percentage of virulence genes as FIM-H 85 (88.5%), *mrk*D 80 (83.4%), *mag*A 22 (22.9%), K2 25 (26.0%), *rmp*A 62 (64.6%), *uge* 74 (77.0%), *kfu* 33 (34.3%), and aerobactin 63 (65.6%).

Virulence genetic characteristics of hv*KP vs* c*KP*. The prevalence of K1 and K2 gene in hv*KP* isolates was significantly increased (P = 0.024 and P = 0.039, respectively), than that in c*KP* isolates. Moreover, hv*KP* strains were strongly associated with *rmp*A (P < 0.001), than c*KP* strains. In addition, *uge*, *kfu* and aerobactin were also strongly associated with hv*KP* strains (P = 0.015, P = 0.014 and P = 0.001, respectively) than c*KP* strains. However, no significant difference was found in FIM-1 and *mrk*D3 genes (P = 0.107 and P = 0.403, respectively) in between hv*KP* and c*KP* strains .

Biofilm and serum resistance characteristics of c*KP vs* ESBL producing c*KP* strains . ESBL producing c*KP* strains were highly associated with strong biofilm formation (P < 0.001) than c*KP* strains. But no significant difference (P = 0.208) was found with moderate biofilm formation in between ESBL producing c*KP* strains and c*KP* strains. In the serum bactericidal test, similar results were found as biofilm formation that ESBL producing c*KP* strains were significantly associated with serum resistance (P < 0.001) than c*KP* strains and no significant difference was found with intermediate sensitive results (P = 0.490) in Table V.

Biofilm and serum resistance characteristics of c*KP vs* hv*KP* strains. The hv*KP* strains were significantly more increased association with moderate and strong biofilm formation (P < 0.001 and P = 0.039 respectively) than c*KP* strains. In addition, the hv*KP* strains were also more associated with intermediate sensitive and resistance of the serum bactericidal test (P = 0.002 and P = 0.004 respectively) than c*KP* strains in Table VI.

Biofilm and serum resistance characteristics of ESBL producing c*KP vs* hv*KP* strains. The hv*KP* strains were only significantly more associated with moderate biofilm formation and intermediate sensitive of serum bactericidal test (P = 0.005 and P = 0.016 respectively) than ESBL producing c*KP* strains. Whereas, no significant difference was found with strong biofilm formation (P = 0.105) and with serum resistance (P = 0.420) of serum bactericidal test in between ESBL producing c*KP* strains and hv*KP* strains, Table VII. Biofilm and serum resistance characteristics of ESBL producing c*KP vs* ESBL producing hv*KP* strains. No significant

Table IV
The distribution of types of ESBL genes in *c*KP strain and *hv*KP strain.

Types of ESBL	*c*KP (Total no. of strains = 28) No. of strains (%)	*hv*KP (Total no. of strains = 11) No. of strains (%)
TEM	1 (3.6%)	2 (18.2%)
SHV	6 (21.4%)	5 (45.4%)
CTX-M	4 (14.3%)	4 (36.3%)
TEM+SHV	1 (3.6%)	–
TEM+CTX-M	11 (39.3%)	–
SHV+CTX-M	1 (3.6%)	–
TEM+SHV+CTX-M	4 (14.3%)	–

Table V
The comparision of biofilm and serum resistance in between *c*KP and ESBL producing *c*KP strains.

	*c*KP (n = 29) No. (%)	*c*KP producing ESBL (n = 28) No. (%)	P – value
Biofilm formation: Non**	21 (72.4%)	4 (14.3%)	0.001*
Moderate	2 (6.9%)	5 (17.9%)	0.208
Strong	6 (20.7%)	19 (67.9%)	0.001*
Serum Res. test: Sensitive	21 (72.4%)	6 (21.4%)	0.001*
Intermediate sensitive	6 (20.7%)	8 (28.6%)	0.490
Resistance	2 (6.9%)	14 (50%)	0.001*

** Non and weak biofilm formation is kept in non biofilm formation result.

Table VI
The comparison of biofilm and serum resistance between *c*KP and *hv*KP strains.

	*c*KP (n = 29) No. (%)	*hv*KP (n = 28) No. (%)	P – value
Biofilm Formation: Non**	21 (72.4%)	0 (0%)	0.001*
Moderate	2 (6.9%)	15 (53.6)	0.001*
Strong	6 (20.7%)	13 (46.4%)	0.039*
Serum Res. test: Sensitive	21 (72.4%)	0 (0%)	0.001*
Intermediate sensitive	6 (20.7%)	17 (60.7%)	0.002*
Resistance	2 (6.9%)	11 (39.3%)	0.004*

** Non and weak biofilm formation was kept in non biofilm formation result.

Table VII
The comparison of biofilm and serum resistance between ESBL producing *c*KP and *hv*KP strains.

	ESBL producing *c*KP (n = 28) No. (%)	*hv*KP (n = 28) No. (%)	P – value
Biofilm Formation: Non**	4 (13.4%)	0 (0%)	0.111
Moderate	5 (17.9%)	15 (53.6%)	0.005*
Strong	19 (67.9%)	13 (46.4%)	0.105
Serum Res. Test : Sensitive	6 (21.4%)	0 (0%)	0.023*
Intermediate sensitive	8 (28.6%)	17 (60.7%)	0.016*
Resistance	14 (50%)	11 (39.3%)	0.420

** Non and weak biofilm formation is kept in non biofilm formation result.

Table VIII
The comparison of biofilm and serum resistance between ESBL producing *c*KP and ESBL producing *hv*KP strains.

	ESBL producing cKP (n = 28) No. (%)	ESBL producing hvKP (n = 11) No. (%)	P – value
Biofilm formation: Non**	4 (14.3%)	0 (0%)	0.309
Moderate	5 (17.9%)	5 (45.5%)	0.080
Strong	19 (67.9%)	6 (54.5%)	0.435
Serum Res. test: Sensitive	6 (21.4%)	0 (0%)	0.158
Intermediate Sensitive	8 (28.6%)	6 (54.5%)	0.128
Resistance	14 (50%)	5 (45.5%)	0.798

** Non and weak biofilm formation is kept in non biofilm formation result.

difference was found in either biofilm formation or bactericidal effect of serum in between ESBL producing c*KP* and ESBL producing hv*KP* strains (both P > 0.05) in Table VIII.

Discussion

Although *K. pneumoniae* is known to be a common pathogen responsible for community and hospital-acquired pneumonia as well as blood and urinary tract infections (Lin *et al*., 2010; Podschun and Ullmann, 1998). Our data demonstrated a negative association between pneumonia and hv*KP* (P = 0.009). This implies that c*KP* strains predominantly are associated with respiratory infections. These data are consistent with previous reports (Li *et al*., 2014). In this study, we compared the drug resistance and ESBL characteristics of hv*KP* and c*KP* isolates. Previous studies have indicated that hv*KP* strains are stronger resistant to antibiotics than c*KP* strains, whereas more recent studies have not only indicated that such strains are less associated with antibiotic resistance (Li *et al*., 2014), but also shown that ESBL were significantly lesser than c*KP* strains (Su *et al*., 2008). Consequently, these data are inconclusive. In the present study, hv*KP* strains were shown less resistant than c*KP* strains, for 10 out of 17 antimicrobial drugs tested. Moreover, ESBL were shown a negative association with hv*KP* (P = 0.40). In addition, the c*KP* strains possessed one to three types of ESBL gene but hv*KP* strains have only one type. The reason for this difference remains unknown. It can be speculated that hv*KP* strains cannot acquire resistance-related plasmids, or that some drug-resistant genes are lost when they become hypervirulent (Li *et al*., 2014). Virulence genetic characteristics of hv*KP vs* c*KP* show That the hv*KP* was more virulent in a model of subcutaneous abscess of rat than c*KP* (Pomakova *et al*., 2012). Serotype K1 and K2 of *K. pneumoniae* cause pyogenic liver abscess and is also repeatedly reported in community acquired pneumonia (Decre *et al*., 2011). The *kfu* which mediates uptake of ferric iron, is more common in hv*KP* compared with c*KP* strains and was shown to be a virulence factor in mice after IG (intragestinal) but not after IP (intraperitonium) (Ko *et al*., 2002). Aerobactin production was more common in hv*KP* strains than c*KP* strains, which was demonstrated by a cross-feeding assay (Yu *et al*., 2007). This analysis suggested that the hv*KP* strains might have the capability to acquire iron more readily than the c*KP* strains.

In this study, we found that the prevalence of virulence associated genes viz., K1, K2, *rmp*A, *uge*, *kfu* and aerobactin were strongly associated with hv*KP* than c*KP* strains. There was no any significant difference of FIM-1, *mrk*D genes between both. These data are consistent with previous reports, which describe an association between K1 and K2 expression and associated with rmpA in between hv*KP* and c*KP* strains (Li *et al*., 2014). Also an agreement with some another studies suggested that the K1 and K2 capsular genes are common in hv*KP* strains, c*KP* strains may also pos-

sess these genes (Brisse *et al*., 2009) and hv*KP* strains may have a non-K1/K2 genes (Fang *et al*., 2007). Some studies have considered the sources of the specimen and found that the proportion of ESBL-producing strains in the isolates from sputum and urine except blood and wound was significantly higher in biofilm-forming strains than in non-biofilm-forming strains (Watnick and Kolter, 2000; Yang and Zhang, 2008). In the present study, we showed that the ESBL producing isolated strains had a more ability to form biofilm in comparison with non ESBL producing strains in the source of sputum specimen. It may be due to: (i) the biofilm is a multispecies microbial community and these species can share at a high rate of their genetic material (ii) the ESBLs can be induced by low concentration of antibiotic, which is a cause of decrease penetration into biofilm (Wacharotayankun *et al*., 1993; Yang and Zhang, 2008). Another recent study identified that the hv*KP* strain produces higher biofilm than c*KP* strains (Wacharotayankun *et al*., 1993). Even our study showed similar correlation between hv*KP strain* and c*KP strain*, and it has been suggested that this ability increases colonization. Studies have shown that serum-resistant strains are significantly more increased among ESBL-producing *K. pneumoniae* strains than among non-ESBL producer strains (Sahly *et al*., 2004) and hv*KP* strains are more resistant to serum than compared with c*KP* strains (Wacharotayankun *et al*., 1993). We investigated a relationship between ESBL production and the serum resistance of hv*KP* strains and c*KP* strains. The ESBL-producing *K. pneumoniae* strains showed significantly more resistant to the serum bactericidal effect than their non-ESBL-producing strains. Moreover, hv*KP* strains were shown significantly high resistant to the serum bactericidal effect than compared with c*KP* strains. However, no any significant difference was found in between ESBL producing c*KP* strains and ESBL producing hv*KP* strains. It has been suggested that (I) the production of R-plasmid-coded ESBLs are increased when associated with adhesive (Darfeuille-Michaud *et al*., 1992), (ii) and the increase production of extra-capsular polysaccharide of hv*KP* strains (Wacharotayankun *et al*., 1993).

Conclusion

The hv*KP* strains produce increased biofilm, less susceptible to serum and possess more virulence gene compared with c*KP* strains. Whereas, the ESBL producing c*KP* strains didn't show any significant difference between biofilm formation and bactericidal effect of serum compared with hv*KP* strains and ESBL producing hv*KP* strains. Thus hv*KP* strains and ESBL producing c*KP* strains are highly pathogenic to compare c*KP* strains.

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Conflicts of interests

We declare that we do not have conflicts of interest

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ORIGINAL PAPER

Characterization of Microbial Communities in Acidified, Sulfur Containing Soils

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Abstract

Over a period of three years, microbial communities in acidified soil with high sulfur content were analyzed. In soil water extracts ureolytic, proteolytic, oxidoreductive, and lipolytic activity were detected. The presented results indicate that the enzymatic activity of soil microbial communities varied considerably over time. Isolated 26 (80%) bacterial strains belonged to genus *Bacillus* sp. and were identified by cultivation and 16S rRNA methods. The commercially available procedures for bacterial DNA isolation from acidified soil failed, therefore a new, specific DNA isolation method was established. Ureolytic activity, detected in soil extracts as well as in isolated *Bacillus* sp. strains may be considered as a tool for the bioremediation of acidified soils with high sulfate content.

Key words: acidified soil microflora, DNA isolation specific method, soil bacteria, soil biochemical activity

Introduction

Soil is a highly heterogeneous microbiome habitat due to the varied physical and chemical conditions of microniches (Ettema and Wardle, 2002; Xu *et al*., 2014). The enzymatic activity found in the soil is mainly of microbial origin, being derived from intracellular, cell-associated or free enzymes. The biochemical activity of soil is a key factor in its natural remediation, and microbial activity acts as a biomarker of soil condition (Kumar *et al*., 2013; Wang *et al*., 2014).

Sulfur mines have a destructive influence on the surrounding biocenoses (González *et al*., 2011). The contamination of soil with sulfur results in a change of groundwater chemistry and plant growth inhibition (Li *et al*., 2006). Technogenic soils derived from sulfur mines present a challenge in terms of rehabilitation processes. Ureolytic activity is essential for the nitrification process, determining the amount of nitrogen compounds available to plants (Cheng *et al*., 2013). In acidic soils, nitrification may be restricted to microniches where active ureolytic bacteria are present at higher pH levels. pH is one of the most essential factors for the growth of soil microorganisms as well as the availability of carbon and nitrogen sources and metal solubility. In another studies of acidified soils, fungi prevail over bacteria and artificial fertilization may increase the urease activity of soils (Rousk *et al*., 2009; Krzywy-Gawrońska, 2012). Kang and co-authors showed the possibility of using ureolytic bacteria for lead removal by biomineralization (Kang *et al*., 2015). Ureolytic *Bacillus* strains may be applied for soil solidification by enhancing the precipitation of $CaCO_3$ (Shirakawa *et al*., 2011).

In the present study we investigated soil samples collected from the vicinity of the former Grzybów sulfur mine located in south-western Poland. The Grzybów sulfur mine extracted sulfur by the hot water method from ore 180 m below sea level in the years 1966–1996 (Zieliński and Wałek, 2012). The high content of sulfur (4%) resulted in lower hygroscopic and capillary capacity of the soil (Zieliński and Wałek, 2012). In 1966 the Grzybów sulfur mine was closed and the surrounding area was subjected to rehabilitation, but those efforts failed and plant vegetation is still absent. Up to date microbial communities of sulfate, acidified soil (pH 4.0) were not investigated.

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Aims of presented studies were to characterize soil microbial communities by their biochemical activities and strains identification. It was done by over the period of three years.

In aqueous extracts from acidified soil samples ureolytic, oxidoreductive, and lipolytic activity were determined. From soil samples, isolated 26 (80%) bacterial strains belonged to genus *Bacillus* sp. that were identified by cultivation and 16S rRNA methods.

Experimental

Materials and Methods

Site description and chemical determination of soil samples. The studied area was located near the town of Staszów (Fig. 1). Samples were collected in October 2010, 2011, and 2012 from the floor of a pine forest and from a plant-free site (sample 5). Weather conditions during sample collection are presented in Table I. The prevalent rocks in the studied region include Miocene limestone, gypsum, clay, and sand, which are covered by Quaternary layers. Terrain is slightly undulating and dotted with karst funnels on top of non-karst layers. In spite of drainage by the river Czarna Staszowska, many small lakes and bogs are present in this area due to karst phenomena. Silty-boggy soils and black soils are found in depressions. Samples of 0–7 cm topsoil were

Table I
Coordinates of sampling sites and weather conditions during sample collection*

Sample number	Sampling place	Elevation (MASL)	Latitude	Longitude
1 (G1)	Rzędów	238	50°55.039’	21°03.223’
2 (G2)	Donica	208	50°55.432’	21°21.053’
3 (G3)	Dziki Staw	182	50°52.405’	21°17.190’
4 (G4)	Jasny	215	50°57.542’	21°24.507’
5 (G5)	Grzybów Pustynia	235	50°52.817’	21°07.396’

October	2010	2011	2012
Monthly average temperature (°C)	5.8	7.8	7.9
Monthly sum of precipitation (mm)	7.2	24.4	102
Monthly humidity (%)	84	85	87

* data from Hydro-Meteorological Station Sukow 19B, 26–021 Daleszyce, Poland, sampling was done in October of each year.

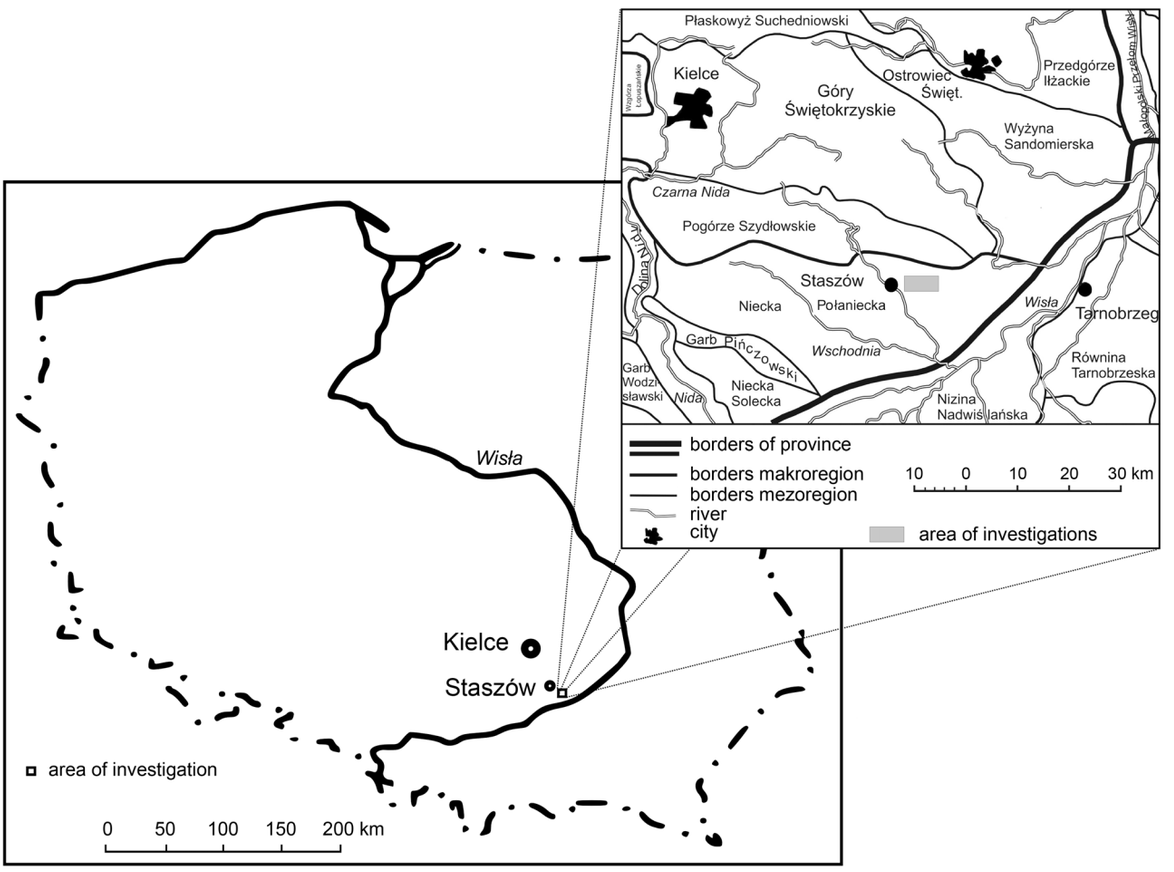

Fig. 1. Map showing the location of Grzybów and the sampling sites. Based on Kondracki maps, 1994 (Zielinski and Walek, 2012).

collected and kept for 24 hours at 4°C under humid conditions till experiments were performed. pH was determined by two methods: in water and in the presence of KCl. Selected physicochemical parameters (pH in H_2O and in KCl, soil organic matter, and carbonate content) were determined in soil samples using standard procedures (Van Reeuwijk, 2006). To determine element concentrations in soil, the samples were dried at ambient temperature, sieved (< 2.00 mm), and disaggregated with a Fritsch mill to pass a 0.063 mm sieve. Homogenized samples were placed in small seal-top polyethylene sample bags and analyzed using a portable XRF analyzer (Niton XL3t from Thermo Scientific) with a geometrically optimized large drift detector and a 50 kV X-ray tube with Ag anode. The soil analytical mode was selected for analysis and each measurement was done in triplicate. The following elements were determined with precision expressed as 2 standard deviations of the mean from three measurements (in parentheses): S (± 200 mg/kg), K (± 200 mg/kg), Ca (± 100 mg/kg), Ti (± 200 mg/kg), V (± 10 mg/kg), Cr (± 20 mg/kg), Fe (± 100 mg/kg), Zn (± 40 mg/kg), As (± 10 mg/kg), Rb (± 0.00), Sr (± 0.00), Zr (± 20 mg/kg), Pb (± 10 mg/kg), Mn (± 10 mg/kg). A certified reference material NIST 2709a (San Joaquin Soil) was used for quality control. Detailed chemical analysis of five sampling sites, over a period of three years are presented in Table III.

Total soil DNA isolation. Total DNA from soil samples was isolated according to the procedure given below: 2 ml of phosphate buffer was added to 1 g of freeze-dried soil and mixed. The mixture was supplemented with 0.4 ml of 5% SDS and 0.04 g of PVPP. Humic acids were precipitated by adding $CaCl_2$ to a final concentration of 2% (w/v). Samples were incubated for 1 h in an incubator at 65°C and centrifuged at 8,000 × g for 10 min at 10°C. The supernatant was collected into new tubes. Then, 570 µl of TE buffer, 30 µl of 10% SDS, and 3 µl of proteinase K (20 mg/ml) were added. The tubes were incubated at 37°C for 1 h. Then, 100 µl of 5 mol/l NaCl was added, followed by vigorous stirring. Subsequently, 80 µl of CTAB solution (10% hexadecyltrimethylammonium bromide in 0.7 mol/l NaCl) was added. In the next step, 700 µl of phenol-chloroform was added, mixed, and supplemented with 30 µl of isoamyl alcohol. The mixture was centrifuged at 20,000 × g at ambient temperature for 3 min. After centrifugation, the aqueous phase was collected and added to the mixture of 700 µl of phenol-chloroform and 30 µl of isoamyl alcohol. The mixture was centrifuged at 20,000 × g at ambient temperature for 3 min. The supernatant was transferred to a new tube and 420 µl of isopropanol was added. The mixture was centrifuged at 20 000 × g at ambient temperature for 3 min. The supernatant was decanted (a mixture of phenol-chloroform and ethanol) and remaining supernatant was removed with a pipette. Next, 100 µl of 70% alcohol was added. The mixture was centrifuged at 15,000 revolutions (20 000 × g) at ambient temperature for 3 min and the supernatant was evaporated. Finally, 100 µl of TE buffer was added.

A second method for total soil DNA isolation was also used. DNA isolation was performed according to the procedure described by Tsai and Olson (Tsai and Olson, 1991). Isolation was followed by DNA purification on a column filled with Sepharose 4B-CL (Sigma) and elution in three centrifugation steps 1000 × g for 2 min with TE buffer. Additionally, total DNA isolation was performed using the commercial kit Genomic Mini AX SOIL Spin (AA Biotechnology).

Ribosomal intergenic spacer analysis (RISA). Primers designed for RISA are complementary to the 16S and 23S rRNA conservative sequences. PCR amplifies the non-coding sequence between those genes. This non-coding sequence accumulates mutations and has different length in each species. RISA analysis was performed on isolated DNA templates from five soil samples. The intergenic spacers between the small- and large-subunit rRNA genes were amplified using the primers S-D-Bact-1522-b-S-20 (eubacterial rRNA small subunit, 5'-TGCGGCTGGATCCCCTCCTT- 3') and L-D-Bact-132-a-A-18 (eubacterial rRNA large subunit, 5'-CCGGGTTTCCCCATTCGG-3') (Ranjard *et al*., 2000). Amplification was performed in an Eppendorf Mastercycler EP at 94°C for 3 min, followed by 25 cycles at 94°C for 1 min, 55°C for 30 s, and 72°C for 1 min; extension of incomplete products was conducted at 72°C for 5 min (Ranjard *et al*., 2000).

DGGE of 16S rRNA fragments. The 16S rDNA fragment was amplified in PCR with primers for the conserved regions of the 16S rRNA genes. The nucleotide sequences of the primers were as follows: V3F 5'-CCTACGGGAGGCAGCAG-3'; V3R 5'-ATTACCGC GGCTGCTGG-3'; and V3FGC 5'-CGCCCGCCGCG CGCGGCGGGCGGGGCGGGGGCACGGGGG GCCTACGGGAGGCAGCAG-3'. The PCR procedure was performed according to the protocol described by Muyzer (Muyzer *et al*., 1993).

Biochemical activity water extracts of soil samples. Determination of the total biochemical activity water extracts of soil samples were performed. Ureolytic activity was determined at 37°C, 25°C and 4°C based on the detection of ammonium ions in a phenol-hypochlorite assay (Moreno *et al*., 2001). Lipolytic activity was determined using p-nitrophenyl butyrate (pNPB) as a substrate (Margesin *et al*., 2002), proteolytic activity was measured in the presence of sodium caseinate (Subrahmanyam *et al*., 2011), oxidoreductase activity with dimethyl-p-phenylenediamine and alpha-naphtol (Kumar *et al*., 2013). Activities were determined at

25°C. Due to the lack of some soil samples not for all periods of time were tests done.

Ureolytic assay. Determination of the ureolytic activity of the bacterial strains isolated from soil samples: liquid Christensen media (pH 4, 5, 6 and 7) were inoculated (1:50) with a bacterial suspension (in sterile saline, McFarland standard 1) and incubated at an appropriate temperature. Change of color (arising as a result of urease activity) was measured with a spectrophotometer (λ = 560 nm) *vs.* a non-inoculated medium.

Soil bacteria isolation. Isolation of soil bacteria: 1 g of each soil sample was suspended in sterile Winogradsky salt solution (0.4 g of K_2HPO_4, 0.13 g of $MgSO_4 \cdot 7H_2O$, 0.13 g of NaCl, 1.52 mg of $MnSO_4 \cdot H_2O$, and 0.5 g of NH_4NO_3) and stirred intensively using a magnetic stirrer for 15 min. After sedimentation of soil particles, the soil solution was diluted to 10^{-6} and spread on the surface of soil extract agar (SEA – 0.5% glucose, 0.5% peptone and 1% soil extract). Each soil extract and its dilutions were plated on separate SEA plates. Plates were incubated at 25°C and 4°C for up to 1 and 2 weeks, respectively, and the number of aerobic bacteria was determined. Bacterial strains with different macroscopic morphologies were isolated and investigated. The nomenclature of the isolated strains in Table II should be read as follows: G1/1 – sample 1, strain 1 with the plate with colonies of similar morphology.

Bacterial DNA isolation. DNA was isolated from bacterial cultures using a Genomic DNA Mini Kit (AandA Biotechnology) according to the manufacturer's instructions (temperature of incubation of a sample with lysis buffer was raised from 37°C to 56°C).

Identification of bacterial strains. Bacteria were identified by sequencing an amplified 16S rDNA fragment. Com1: 5'-CAGCMGCCGCGGTAATWC and Com2: 5'-CCGTCAATTCMTTTRAGTTT primers (Schwieger and Tebbe, 1998) were used for PCR at a concentration of 0.2 μM each. Approximately 0.1 ng of bacterial DNA was used as a template. The reaction was done with a DreamTaq™ Green Master Mix (Fermentas). Reaction mixtures were placed at 94°C for 5 min and then subjected to 30 cycles of amplification by incubation at 94°C for 2.5 min, 55°C for 30 s and 72°C for 2 min. Finally, incubation was conducted at 72°C for 5 min. The quality of the obtained PCR products was tested by electrophoresis on 2% agarose, followed by staining

Table II
Ureolytic activity of bacterial strains isolated from acidified, sulphate soils.

Year	Soil	Temperature +25°C								
2011	G1	G1/1	G1/2a	G1/2b	G1/3	G1/4	G1/5	G1/6	G1/8	G1/17
		G1/9	G1/10	G1/11	G1/12	G1/13	G1/14	G1/15	G1/16	
	G2	G2/2				G2/4				
	G3	G3/1			G3/2a			G3/2b		
		G3/3			G3/4			G3/5		
		G3/6			G3/7			G3/8		
	G4	G4/2		G4/9		G4/11			G4/15	
		G4/17		G4/18		G4/20			G4/21	
	G5	G5/1				G5/2				
2012	G1	G1/25	G1/26	G1/28		G1/29	G1/30		G1/31	
		G1/33a	G1/33b	G1/34a		G1/34b	G1/35		G1/36	
		G1/38	G1/39	G1/40		G1/41		G1/43	G1/46	
	G2	2/14								
	G3	G3/10		G3/12		G3/16			G3/21	
	G4	G4/36		G4/40		G4/43			G4/46	
	G5	-								

Legenda:

Ureolytic activity	Scale
None	
Weak	
Moderate	
High	

with ethidium bromide. Subsequently, PCR products were sequenced using a Beckman-Coulter CEQ™ 8000 Genetic Analysis System. 16S rDNA sequences were identified based on data from the Ribosomal Database Project, release 10 (http://rdp.cme.msu.edu/).

Determination of the total ureolytic activity of soil samples: ureolytic activity was determined based on detection of ammonium ions in a phenol-hypochlorite assay.

Influence of ureolytic soil bacteria on soil pH. To 5 g of an autoclaved soil sample, 2.5 ml of *Staphylococcus* sp. (sample soil G1) suspension (0.5 McFarland) in sterile saline with 0.4% of urea was added. Parallel samples without bacteria were used as a controls. After incubation for 2 weeks at 25°C, 5 ml of distilled water was added to each sample and pH was measured.

Results

Soil chemical properties. In the tested soil samples, pH varied from 4 (sample 1) to 2.4 (sample 5). In both methods used (H_2O and KCl) the pH of soil samples was found to be highly acidic (Table III). Organic matter content was the highest in sample 3 (27.6%). The highest level of organic carbon detected in sample 3 (near Donica Lake) may be connected to the lay of the land as this site is located in a deep karst funnel and the steeply inclined slope facilitates the retention of organic matter (Tables I and Fig. 1). This was confirmed by the lowest ash content among the sampling sites (Gąsiewicz *et al*., 2012). Sulfate content was below 1% and varied within sampling sites. For example, over a period of 3 years, in sample 1 it was 0.2% in the 1st year, followed by a tenfold decrease in the 2nd and 3rd years. This variation was not observed in sample 4. As concerns metal content, calcium was the most abundant, and again it varied over time, but less significantly than sulfates. Trace amounts of other metals, including chromium, manganese, and vanadium, were detected (Table III).

Biochemical activity of water soil extracts. Ureolytic activity was present in almost all samples (except from sample 5 in the 3rd year – see Table IV). Ureolytic activity was measured at three different temperatures – 37°C, 25°C and 4°C. The highest overall 13.1 nM NH_4 g^{-1} dry soil h^{-1} ureolytic activity was observed in the 1st year, in sample 1 (G1) at 37°C. At 25°C and 4°C water soil extracts ureolityc activities were low in range 2.1 nM NH_4 g^{-1} dry soil h^{-1} (data not shown). Proteolytic activity was detected in all five samples in the 2nd year, but only in sample 1 in the 3rd year. Also lipolytic activity was observed in all samples except for samples 3 and 4 in the 1st year. Differences in enzymatic activities of soil water extracts might be resulted by different monthly precipitation rates in studied areas – see Table I.

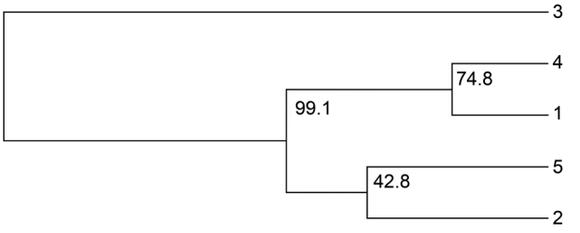

Fig. 2. Phylogenetic tree illustrating the genetic distance between DNA fragments of five soil samples subjected to RISA.

Soil DNA isolation and the presence of a urease operon fragment in soil samples. The commercially available kit did not make it possible to obtain bacterial DNA from acid sulfate soils at an amount and purity suitable for PCR reactions. After several attempts, a modified method was established. A combination of several techniques led to obtaining non-degraded, high molecular weight DNA suitable for molecular analysis (see the Materials and Methods section). To confirm the presence of a urease operon fragment in the tested soil samples, PCR was performed using primers (UreUnR and UreUnF) recognizing the urease-conservative fragments of Gram-negative bacteria. Electrophoresis confirmed the presence of a product of expected length of 440 bp in all soil samples, except for 4 data not shown.

Ribosomal intergenic spacer analysis (RISA). Ribosomal intergenic spacer analysis allows one to estimate the genetic distance between the tested organisms based on the number of and distance between PCR products (Fig. 2). Out of the three DNA isolation methods used, only one method (with PVPP) gave templates sufficiently free from humic acids to be used for PCR. Sample 3 is more distant from other samples, which are clustered in a different clade (Fig. 2). There is a small genetic distance between samples 2 and 5, which suggests that their composition is very similar. Also samples 1 and 4 exhibited a low genetic distance.

DGGE of 16S rRNA fragments. Denaturing gradient gel electrophoresis (DGGE) makes it possible to distinguish between products of the same length but with small different in sequences. Figure 3 presents 16S rRNA analysis from five soil samples. These results confirm RISA analysis that microbiome communities of tested soils are very homogenous and only from 1 to 3 PCR products are detected (Fig. 3).

Isolation, identification and biochemical activity of bacterial species. Over a period of three years, 118 bacterial strains were isolated by the plate method supplemented with a water soil extract. After isolation, only 26 strains were successfully cultivated for a prolonged period of time. The isolated strains were subjected to 16S rRNA analysis. The majority of these strains belong to the family *Bacillaceae* (Fig. 4). Isolated

Table III
Chemical analysis of soil sampling in 2010 (white), 2011 (grey) and 2012 (dark grey)

Sample	pH H_2O/KCl	m_{org}	P	C_{org}	$CaCO_3$	[%]													
						S	K	Ca	Ti	V	Cr	Fe	Zn	As	Rb	Sr	Zr	Pb	Mn
1						0.226 ± 0.03	0.587 ± 0.018	0.692 ± 0.01	0.159 ± 0.06	0.006 ± 0.02	0.002	0.698 ± 0.01	0.08 ± 0.01	0.002 ± 0.01	0.002	0.004	0.011	0.011	0.031
						<0.015	0.929 ± 0.021	0.187 ± 0.01	0.281 ± 0.01	0.008	0.003	0.344 ± 0.01	0.002	< 0.001	0.003	0.004	0.033	< 0.001	0.009
	4.00/3.37	2.9	97.1	1.55	0	<0.015	1.01 ± 0.02	0.114 ± 0.01	0.311 ± 0.01	0.008	0.004	0.478 ± 0.01	0.002	< 0.001	0.004	0.004	0.046	0.002	< 0.001
2						0.067 ± 0.02	0.784 ± 0.019	0.291 ± 0.01	0.230 ± 0.01	0.006	0.002	0.480 ± 0.01	0.004	0.001	0.002	0.003	0.022	0.003	0.022
						0.068 ± 0.04	0.802 ± 0.026	1.89 ± 0.01	< 0.05	0.009	0.005	1.27 ± 0.02	0.007	0.001	0.003	0.006	0.014	0.003	< 0.001
	3.7 0/3.35	6.5	93.5	4.35	0	<0.015	1.06 ± 0.02	0.167 ± 0.01	0.318 ± 0.01	0.009	0.004 ± 0.01	0.461 ± 0.01	0.003	< 0.001	0.004	0.004	0.039	0.003	0.009
3						0.094 ± 0.02	0.496 ± 0.009	0.370 ± 0.009	0.102	0.003	< 0.001	0.361 ± 0.01	0.004	0.001	0.001	0.002	0.005	0.001	0.008
						0.056 ± 0.03	0.537 ± 0.021	0.297 ± 0.01	0.198 ± 0.01	0.005	< 0.001	0.322 ± 0.01	0.004	0.01	0.001	0.001	0.004	0.02	< 0.001
	3.50/3.59	27.6	72.4	16–27.1	0	0.067 ± 0.03	0.753 ± 0.024	0.091 ± 0.01	0.3 ± 0.01	0.013	0.006 ± 0.02	0.706 ± 0.01	0.003	0.001	0.003	0.002	0.008	0.003	< 0.001
4						0.229 ± 0.03	0.356 ± 0.014	1.41 ± 0.02	0.075	0.004	0.009	0.590 ± 0.01	0.015	0.001	0.001	0.003	0.004	0.004	0.025 ± 0.01
						0.057 ± 0.02	0.383 ± 0.016	0.094 ± 0.01	0.098	0.004	< 0.001	0.433 ± 0.01	0.003	< 0.001	0.001	0.002	0.007	0.003	< 0.001
	3.59/3.2	4.7	95.3	11.1	0	0.057 ± 0.02	1.32 ± 0.03	0.131 ± 0.01	0.333 ± 0.01	0.008	< 0.001	0.373 ± 0.01	0.002	0.001	0.004	0.004	0.025	0.004	< 0.001
5						0.666 ± 0.04	0.849 ± 0.018	0.395 ± 0.01	0.292 ± 0.01	0.006	0.002	0.112 ± 0.01	< 0.001	< 0.001	0.002	0.005	< 0.001	0.001	< 0.001
						7.81 ± 0.1	0.883 ± 0.02	0.220 ± 0.01	0.158 ± 0.01	0.004	0.01	0.171 ± 0.01	< 0.001	< 0.001	0.004	0.006	0.009	0.006	> 0.001
	2.41/2.29	1.8	98.2	1.07	7.3	0.550 ± 0.04	0.938 ± 0.02	0.2 ± 0.01	0.302 ± 0.01	0.007	0.004	0.18 ± 0.01	< 0.001	< 0.001	0.004	0.005	0.037	0.005	< 0.001

M_{org} – organic matter content; P – ash content; C_{org} – organic carbon content

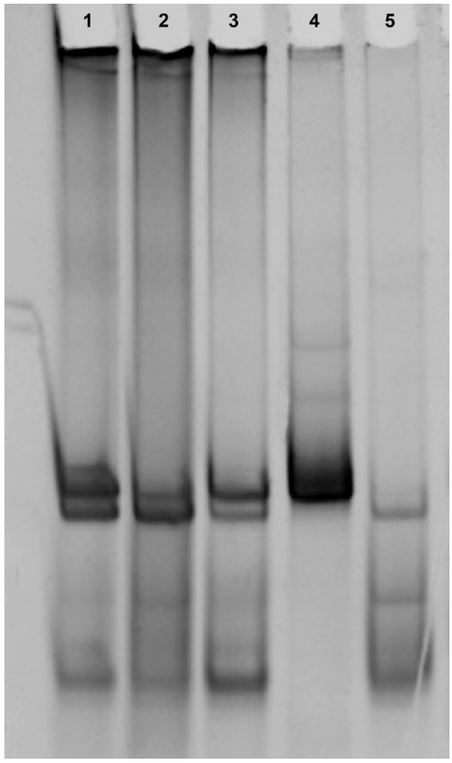

Fig. 3. PCR products of 16S rRNA on a template of five soil DNA isolates (wells 1–5) separated by denaturing gradient polyacrylamide gel electrophoresis (DGGE).

bacteria were grown at both 4°C and 25°C. Staining of the strains isolated in 2011 and 2012 revealed similar results: 75% were Gram-positive and 25% were Gram-negative bacteria. Morphological analysis of Gram-positive bacteria revealed that 60% of them were rod-shaped and 7% were coccoid. *Streptomyces* cells were identified in 2% of samples. The highest number of different species was isolated from sample 1.

It was found that 29 of the tested strains possessed ureolytic activity (Table II). Only one strain could decompose urea at +4°C. Soil sample 5 exhibited a lack of living bacteria with ureolytic activity. A comparison of samples from the 2nd and 3rd years indicated that sample 1 contained the greatest proportion of strains with urease activity (12 strains). Overall, 29 out of 118 strains possessed measurable ureolytic activity. The ureolytic activities of isolated strains indicate that detected in water soil extracts urea decompositions are due to bacterial biochemical activities.

Influence of ureolytic soil bacteria on soil pH. Soil samples inoculated with *Staphylococcus* sp. strain (previously isolated from a soil sample G1) were fertilized with urea and incubated for two weeks. pH levels were measured before and after incubation. In all samples bacteria survived low soil pH and started to decompose urea to carbon dioxide and ammonia, which resulted in an overall weak pH increase in all the soil samples studied, from 0.02 to 0.16 for soil sample G1, G3, respectively.

Discussion

One of the most important environmental factors determining soil bacterial activity is pH. The presented study analyzed soil samples of pH ranging from 4 to 2 from the area of a former sulfur mine, in south-western Poland. The biochemical activities of the bacteria were detected in tested soil samples. Those activities varied, depending on the year of sampling and sample type. The ureolytic activity of soil extracts as well as isolated soil strains was investigated in more detailed due to the expectation that urea decomposition may help to increase very low pH of sulfate soils. The isolated strains that exhibited ureolytic activity were present in all tested soil samples. Low pH is a crucial environmental factor affecting the process of nitrification and inhibiting the decomposition of organic matter as a source of soluble soil nitrogen for plant vegetation (Cui *et al*., 2013). Data suggest that inhibition of microbial species was observed below pH 4.5, which is probably attributable to increased inhibitory effects caused by the release of free aluminum or decreasing

Table IV
Biochemical activities of water extracts of soil samples

	Lipolytic activity (μg p-nitrophenyl butyrate g^{-1} h^{-1})			Proteolytic activity (μg tyrosine g^{-1} h^{-1})		Oxidoreductase activity (U)*	Ureolytical activities (nM NH_4 g^{-1} dry soil h^{-1} at 37°C)		
Year	1st	2nd	3rd	2nd	3rd	2nd	1st	2nd	3rd
Sample 1 (G1)	39.1	17.6	536.9	59.3	21.0	0.16 ± 0.05	13.1	2.2	0.2
Sample 2 (G2)	33.1	1299.8	536.8	80.2	0	0.22 ± 0.02	0.7	3.1	0
Sample 3 (G3)	0	38.6	291.7	77.8	0	0.22 ± 0.04	11.1	1.8	0.1
Sample 4 G4)	0	2299.3	240.6	76.3	0	3.28 ± 0.39	1.7	2.8	0.1
Sample 5 (G5)	94.2	2455.8	0	99.5	0	1.63 ± 0.12	0	2.4	0

* standard deviation

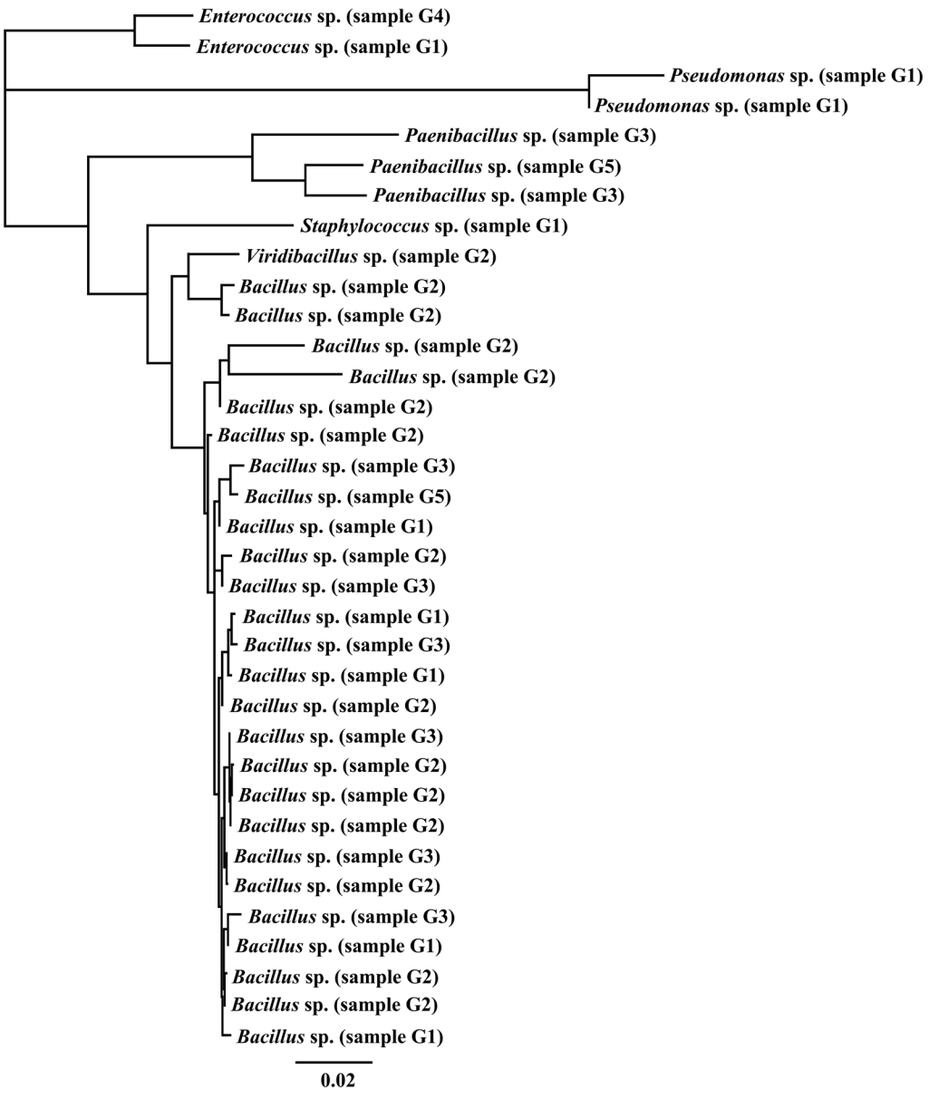

Fig. 4. Identification and phylogenetic tree of bacterial strains isolated from acidic soil samples based on 16S rDNA.

plant productivity (Rousk *et al*., 2009). Bacterial communities in acidic soils are more strongly influenced by low pH than fungal communities (Rousk *et al*., 2010). That might be one cause of failed attempts to rehabilitate the soil by liming the surface, leading to the formation of carbonates and the absence of vegetation in the sampling sites, which is still observed (Zieliński and Wałek, 2012). The majority of bacterial strains isolated from acidified sulfate soils belong to the family *Bacillaceae*. This may indicate that under harsh conditions involving acidic pH and a high content of sulfur only spore-forming bacteria may be able to survive. That observation was confirmed by other studies (van Elsas *et al.,* 2008; Blagodatskaya and Kuzyakov, 2013; Valenzuela *et al*., 2006). Moreover, *Bacillus* spp. are ureolytic strains which can survive acidic conditions (Mols and Abee, 2008). The RISA test was conducted for the isolated soil DNA by our new established method. The low number of DNA bands indicated very low heterogeneity of bacterial populations in acid sulfate soil. As it was aptly stated by Fierer and Jackson, "the diversity and richness of soil bacterial communities differed by ecosystem type, and these differences could largely be explained by soil pH" (Fierer and Jackson, 2006). In general, bacterial diversity in acidic soils is significantly lower than in neutral soils (Fierer and Jackson, 2006). The highest sulfur concentration (7.81% in sample 5 in the 2nd year) exceeded the highest content of this element found by Martyn *et al*. in the soils of the former Basznia sulfur mine in eastern Poland (1.56%) (Martyn *et al*., 2004). The results of sulfur determination in soil samples in conjunction with pH values were similar to those obtained by Sołek-Podwika and Ciarkowska in their comprehensive study of soils in the Grzybów

mining area (Sołek-Podwika and Ciarkowska, 2012). Those authors also observed increasing sulfur concentrations with sampling depth. The relatively low concentrations of metals that were found in the soil samples examined may be explained by the migration of ions mobilized in an acidic environment.

Conclusions

Biochemical activities, based on presence of bacteria, were detected in acidifed sulfate soils. DNA analysis of soil samples indicate on very small diversity of bacterial population, reduced to spore forming *Bacillus* sp. In all the tested samples soil ureolytic activities, the presence of ureolytic bacteria and urease genes were detected. Our study indicates that *Bacillus* sp., strains isolated from acidified sulfate soils may be considered tools for biomineralization that will led to pH increase by urea decomposition.

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ORIGINAL PAPER

Study of Patterns and Markers of Human Immune Deficiency Virus -1 (HIV-1) Progression and Unemployment Rate among Patients from Alexandria, Egypt

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Abstract

Middle East and North Africa (MENA) new HIV cases show the highest increase among all regions in the world. Even though Egypt has a low prevalence among the general population (< 0.02%), a national HIV epidemic occurs in certain population risk groups. The current study was conducted to asses clinical and immunological disease progression; following up viral load (VL) and detecting delta-32 CCR5 genotype polymorphism in selected cases, determining unemployment rate and identify predictors of employment for HIV-cases. A cross sectional design was adopted. HIV infected cases attending Alexandria Fever Hospital (AFH) for one year. Interview questionnaire and four CD+4 counts were done for all patients, HIV VL and delta-32 CCR5 polymorphism were done for selected cases. Sexual transmission and drug abuse are the most important risk factors. Infectious comorbidity increases the rate of HIV progression. CD4+ count at the end of the study; CD+4 (4), count was significantly higher than all other CD4+ readings among the whole cohort and among the treated group. Also, VL at the end of the study; VL(2), was significantly higher than VL(1) among the untreated group. Unemployment rate was 40%. Male gender and obtaining vocational training were significant predictors of employment. It can be concluded that having a family member living with HIV and drug abusers are high risk groups for HIV acquisition. Factors responsible for progression of HIV should be further investigated. Antiretroviral therapy is very effective in checking HIV replication rate, delaying the progression of HIV, reconstituting the immune response and should be available for all cases detected.

Key words: Delta-32 CCR5 sequencing, HIV progression patterns, HIV-RNA viral load, HIV virus, unemployment rate among HIV cases

Introduction

Globally people living with HIV (PLHIV) numbered around 36.7 million at the end of 2015 according to the latest United Nations Acquired Immune Deficiency Syndrome (UNAIDS) 2016 data covering 160 countries (Global AIDS Update 2016 | UNAIDS, 2016). The burden of the epidemic continues to vary considerably between countries and regions. New HIV infections in the Middle East and North Africa (MENA) region have increased by 31% since 2001, which is the highest increase among all regions in the world (Gokengin *et al.*, 2016).

In Egypt, the estimated number of PLHIV is around 11,000 individuals (7,000–19,000) with overall low prevalence among the general population (< 0.02%). Greater Cairo and Alexandria; which have about one third of the population of the country, account for almost two thirds of all HIV infected patients detected. Egypt has a national epidemic concentrated in two key populations; people who inject drugs (PWID) and men who have sex with men (MSM) (Egypt National AIDS program NAP, 2015). Egyptian National guidelines on clinical care and antiretroviral therapy (ART) states that Care begins with CD4 cell count testing and those with CD4 count of 500 or lower are eligible for ART (National Guideline on Clinical Care and Antiretroviral Drugs for Treating and Preventing HIV Infection, Egypt, 2014).

HIV has a variable rate (spectrum) of progression in infected people. This variation in disease development is one parameter that led to the classification of patients as rapid progressors (RPs), slow/intermediate progressors, controllers; elite controllers (EC), viraemic controllers

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(VC) and long-term nonprogressors (LTNPs). The classification is also based on CD4 count, HIV viral load (VL), and ART. Different studies suggested different definitions to these subgroups (Gurdasani *et al.*, 2014).

Understanding mechanisms for significantly delaying disease progression remains a worthwhile research goal. Nonprogression probably results from multiple virologic, immunologic, and genetic factors. In terms of host genetics, some LTNPs display polymorphisms in chemokine receptors (CCR5 and CXCR4) and chemokines (*e.g.* MIP-1α and RANTES). Nearly 11% of Caucasians and 2% of Blacks are homozygous for the CCR5-delta32 mutation (Poropatich and Sullivan, 2011).

Employment status is a major predictor of health status and living conditions. However, unemployment rate among HIV – cases ranges from 45% to 65%. It is still higher compared with the general population, in many countries including highincome ones (Legarth *et al.*, 2014). Women, younger age, poor educational level, progressed HIV infections, or Hepatitis C virus (HCV) co-infection are risk factors for unemployment among HIV-cases (Gros *et al.*, 2016).

Our study aimed at (1) assessing clinical and immunological disease progression; (2) evaluating and following up the VL of selected cases, (3) detecting delta-32 CCR5 genotype polymorphism in selected cases, (4) determining unemployment rate and identify predictors of employment for HIV-cases.

Experimental

Material and Methods

A cross sectional design was adopted. All HIV infected cases attending Alexandria Fever Hospital (AFH) during field work period of the study from January to December 2015, who agreed to participate in the study were included (n = 150). Patients were excluded if they were younger than 18 years or refused to participate. They were diagnosed earlier by AFH protocol for HIV diagnosis (National Guideline on Clinical Care and Antiretroviral Drugs for Treating and Preventing HIV Infection, Egypt, 2014).

Study tools. An interview questionnaire. A predesigned questionnaire was used to obtain data from all HIV infected patients (n = 150) concerning a) sociodemography, b) risk factors, c) health status, d) history of the disease and e) treatment regimens of the participants.

Laboratory investigations

Blood samples were collected and analyzed at the AFH and Alexandria Faculty of Medicine (AFM) Central Laboratories.

The immunological status of the participants (CD4 count). CD4 + T-cell count (cells/mm^3) was taken as an immunological marker in the course of disease progression. Five CD4 counts were performed for all patients (n = 150): CD4+ (0); earliest CD4+ T-cell count (on diagnosis of HIV), CD4+ (1); CD4+ measured at the beginning of the study, CD4+(2); CD4+ measured after 3 months of the study, CD4+(3); CD4+ measured after 6 months of the study, CD4+(4); CD4+ measured after 9 months of the study.

Whole blood (200 µl) was collected in EDTA vacutainer tubes (Becton-Dickinson Mt View, California,). The PartecCyFlow Counter® (Partec GmbH, Munster, Germany) for CD4+ T-cell enumeration was used for performing the CD4 count. The Partec CD4% Reagent kit consists of MEM-24; a monoclonal antibody, which recognizes human CD4 antigen (Manasa *et al.*, 2007).

HIV viral load. VL was done twice to selected cases (n = 20). First VL (VL1) was done at the beginning of the study and the second VL (VL2) was done after one year. In our study we were more puzzled by PLHIV not showing any sign or symptoms for variable duration of time despite of not receiving treatment. So, we choose 16 out of the 29 untreated cases in our cohort to be followed up according to their VL in conjunction with their CD4 count. The other 13 untreated cases were excluded for the following reasons, presence of co-morbid infective diseases (HBV/HCV), noncompliance, and unwillingness to participate. In scope of covering the whole spectrum of different patterns of progression, 4 treated interesting cases were also chosen. A case with CD4 rapid decline after HIV diagnosis that dramatically improved on ART. The parents; diagnosed 17 years ago, of a family with four children, all are PLHIV. Lastly a case diagnosed and untreated for 5 years ago then underwent treatment when its CD4 started to decline.

Plasma were separated from the blood samples within 4 hours of collection and stored in a −20°C freezer. RNA extraction was performed using Qiagen QIAamp viral RNA mini spin protocol according to manufacturer instructions.

The VL for each of these samples was determined using a US FDA-approved kit (Artus HIV-1 RG RT-PCR kit; Qiagen, Germany).

As described by Luft, 2011; primers were diluted to a stock concentration of 200 mM and stored at −20°C. A concentration of 2 mM for SYBR Green-based reactions was done. A final primer concentration of 10 picomoles/ul was able to provide high-quality and consistent results.

Gene specific primers for the most conserved region of HIV-1 gag gene were chosen (Genbank)
Forward: 5'-ACATCAAGCAGCCATGCAAAT-3',
Reverse: 5'-TACTAGTAGTTCCTGCTATGTC-3'.
Biosystems StepOne™ Real Time PCR system was used

with the following conditions: one cycle of reverse transcription at 45°C for 30 min, followed by one cycle of polymerase activation at 95°C for 5 min then 40 cycles of PCR amplification. Each cycle consisted of denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 30 s.

CCR5 genotyping. Eight cases that fulfilled the criteria of being LTNPs (according to CD4 count, VL, duration since diagnosis and never on ART) were chosen for genotyping for the CCR5-delta32-allele.

After the genomic DNA was extracted using QIAGEN-Blood-Midi-Kit (Qiagen, Germany), screening of the cases for the CCR5-delta32-allele was performed with a genomic PCR using primers flanking the site of the deletion (forward: 5'-CTCCCAGGAATCATCTTTAC-3', reverse: 5'-TCATTTCGACACCGAAGCAG-3'). The PCRs were performed in 50-µl volumes, each comprising 2× mixture containing 1.5 mM $MgCl_2$, 10 mM dNTPS, 2.5 U Taq DNA polymerase, 50 ng genomic DNA, and 20 µmol of each primer (Gero, 2011).

The amplification conditions were as follows: denaturation at 94°C for 1 min, followed by 35 cycles of PCR amplification. Each cycle consisted of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. Following the 35 cycles of PCR there was an extension for 10 min at 72°C.

Results were confirmed by direct sequencing using the BigDye® - Terminator - 1.1. - Cycle-Sequencing - Kit (Applied Biosystems, Germany).

Statistical analysis of the data. The collected data were coded and typed onto computer files. All analyses were performed using SPSS/Pc + software program version 20.0 (Kirkpatrick and Feeny, 2013). The level of significance selected for results was 5% ($\alpha = 0.05$). Analytic statistics were carried out using Chi-Square test (X^2), Fisher's exact test and Student t-test were used to reveal association between characteristics of HIV-infected patients and HIV progression.

Wilcoxon Signed Ranks test, Paired t-test were done to compare mean values of HIV immunological and virological markers reported at different points of time. Additionally, unemployment rate was calculated. Univariate analysis was conducted where basic sociodemographic and health status characteristics were examined for association with respect to employment status, using Chi-Square test (X^2), Fisher's exact test, and Monte Carlo test for categorical variables, and Student t-test for quantitative variables. All variables that were significantly associated with employment status in univariate analysis, were included in multivariate analysis using logistic regression model to investigate predictors of employment among the studied HIV-cases.

Ethical clearance. The protocol of the current study was approved by AFH, MOH and the Medical Research Ethics Committee at AFM. Objectives of the study, expected benefits, types of information to be obtained, procedures, and publication were explained to each participant and an informed written consent was obtained before participation in the study. Moreover, confidentiality of data was insured.

Results

Characteristics of the studied HIV-infected patients with respect to disease progression (n = 150). Two thirds of our study HIV infected patients were males (n = 100, 66.7%). Their age ranged from 18–62 years with mean age of (33.93 ± 9.25) years. The majority of our patients have urban residence (n = 142, 94.7%) with 46 of them (30.7%) clustered in Amryia district. As regarding HIV acquisition risk factors, family member living with HIV was present in 55.3% of cases (n = 83), of which 67.46% (n = 46) have a spouse having HIV. Addiction was found in about 29.3% (n = 44) while 24% (n = 36) were intravenous (IV) abusers.

Regarding HIV progression, based on CD4 count, duration since diagnosis and ART, RP accounted for 56% of the studied group followed by progressors, controllers and LTNP accounting for 24.7%, 12.7% and 6.7% respectively. Among the studied group 24.6% (n = 37) had infectious comorbidity (Hepatitis B and HCV). In the treated group (80%, n = 121), Truvada + Efavirnez was the treatment regimen in 71.1% of cases and Efavirnez + Zidavudine + Lamivudine in 28.9% in cases with comorbidity.

Among different parameters, the presence of comorbidity was significantly associated with disease progression ($X^2 = 6.61$; $p = 0.01$) (Table I).

Immunological makers; CD4 + T-cell count (cells/mm³) among the studied HIV-infected patients. CD4 + (4) was significantly higher than the other four readings [(CD4 + (0), CD4 + (1) CD4 + (2), CD4 + (3)] among all HIV infected patients; n = 150, and also among the treated group; n = 121, ($p = 0.00$ and $p = 0.00$ respectively) (Table II).

Viral Load – VL (IU/ml) among the studied HIV-infected patients. VL was taken as a virological marker in the course of disease progression. Twenty cases that were chosen, showing different patterns of progression; one was RP, three intermediate were progressors, eight were controllers, 8 were LTNPs. Two LTNPs cases had VL BDL (< 50 IU/ml). VL(2) was significantly higher than VL(1) when assessing patients with detectable VL; n = 18, and also when assessing untreated patients with detectable VL; n = 14, ($p = 0.00$ and $p = 0.00$ respectively) (Table III).

CCR5 genotyping. PCR fragment of 200 base pairs (bp) were detected for CCR5 gene. The eight LTNP selected cases were found to carry the wild type of CCR5.

Table I
Characteristics of the studied HIV-infected patients with respect to disease progression (n = 150)

Characteristics	Non-progressive[$] (n = 29)		Progressive[^] (n = 121)		Test of significance (*P* value)
	No.	(%)	No.	(%)	
Gender Male	19	(65.5%)	81	(66.9%)	$X^2 = 0.02$ (0.88)
Female	10	(34.5%)	40	(33.1%)	
Age (Years) (Mean ± SD)	33.97 ± 11.06		33.93 ± 8.81		t = 0.02 (0.98)
Residence Rural	2	(6.9%)	6	(5%)	[FE]P = 0.65
Urban	27	(93.1%)	115	(95%)	
History of blood transfusion	4	(13.8%)	13	(10.7%)	[FE]P = 0.74
Family member living with HIV	14	(48.3%)	69	(57%)	$X^2 = 0.72$ (0.39)
IV drug abuser	6	(20.7%)	30	(24.8%)	$X^2 = 0.21$ (0.64)
Employment status Unemployed	12	(41.4%)	48	(39.7%)	$X^2 = 0.02$ (0.86)
Employed	17	(58.6%)	73	(60.3%)	
Co-morbidity None	26	(89.7%)	79	(65.3%)	$X^2 = 6.61$ (0.01)*
Infectious[<] and/or non-infectious[#]	3	(10.3%)	42	(34.7%)	

Abbreviations: HIV – human immune deficiency virus; IV – intravenous; [#] – diabetes Mellitus, hypertension;
[<] – hepatitis C virus, hepatitis B virus; [$] – includes long-term non-progressive and controller;
[^] – includes progressive and rapidly progressive; X^2: Chi square test; FE: Fisher's Exact test; t: student t test;
SD: standard deviation; * – significant at p ≤ 0.05 (2-tailed)

Table II
Immunological makers; CD4 + T-cell count (cells/mm^3) among the studied HIV-infected patients

CD4 + T-cell count among all HIV-infected patients (n = 150)			
	Min-Max	Mean ± SD	Test of significance (p-value)
CD4+ (0)	28 – 1555	438.35 ± 259.15	Z" = –8.66 (0.00)**
CD4+ (1)	28 – 1555	474.14 ± 294.32	Z[$] = –8.11 (0.00)**
CD4+ (2)	48 – 1560	484.50 ± 283.11	Z[^] = –8.48 (0.00)**
CD4+ (3)	67 – 1500	495.72 ± 279.55	Z[@] = –7.95 (0.00)**
CD4+ (4)	74 – 1523	506.45 ± 279.21	
CD4 + T-cell count among HIV-infected patients who received treatment (n = 121)			
	Min-Max	Mean ± SD	Test of significance (p-value)
CD4+ (0)	28 – 732	360.60 ± 166.30	t" = –7.61 (0.00)**
CD4+ (1)	28 – 815	367.09 ± 170.26	t[$] = –10.06 (0.00)**
CD4+ (2)	48 – 820	382.83 ± 166.35	t[^] = –7.91 (0.00)**
CD4+ (3)	67 – 859	397.23 ± 168.15	t[@] = –5.46 (0.00)**
CD4+ (4)	74 – 836	407.27 ± 165.62	

Table II
Continued

CD4 + T-cell count among HIV-infected patients who did not receive treatment (n = 29)			
	Min-Max	Mean ± SD	Test of significance (p-value)
CD4+ (0)	369 – 1555	762.72 ± 322.13	Z" = –1.82 (0.06)
CD4+ (1)	369 – 1555	920.79 ± 283.90	t[$] = 0.02 (0.97)
CD4+ (2)	412 – 1560	908.72 ± 277.28	t[^] = –0.58 (0.56)
CD4+ (3)	481 – 1500	906.65 ± 279.10	t[@] = –1.12 (0.27)
CD4+ (4)	521 – 1523	920.27 ± 278.25	

Abbreviations:
HIV – human immune deficiency virus;
Z – Z value of Wilcoxon Signed Ranks test; t: paired t-test;
D: standard deviation; ** – significant at p ≤ 0.01 (2-tailed);
" – CD4+ (0) – CD4+ (4); [$] – CD4 + (1) – CD4 + (4);
[^] – CD4 + (2) – CD4 + (4); [@] – CD4 + (3) – CD4 + (4);
CD4 + (0) – earliest CD4 + count;
CD4 + (1) – CD4 + measured at the beginning of the study;
CD4 + (2) – CD4 + measured after 3 months of the study;
CD4 + (3) – CD4 + measured after 6 months of the study;
CD4 + (4) – CD4 + measured after 9 months of the study

Characteristics of the studied HIV-infected patients with respect to patients' employment status (n = 150). Unemployment rate among HIV-infected patients was 40%. Most of employed HIV-infected patients were men (85.6%), while most of unemployed group were women (61.7%) ($X^2 = 36.12$; p = 0.00). Additionally, 41.1% and 6.7% of employed HIV-infected patients obtained vocational training and secondary school education respectively, compared with unemployed group (11.7% and 5% respectively) ([Monte Carlo] p = = 0.00). Significantly higher percentage of unemployed HIV-infected patients had family member living with

Table III
Viral load (IU/ml) among the studied HIV-infected patients

	VL among all HIV-infected patients (n = 20)			
	No.	%	Mean ± SD	Test of significance (p value)
VL (1) < detection level[d]	2	10	–	$Z^{\&} = -2.84$ (0.00)**
> detection level[d]	18	90	6990 ± 8593.65	
VL (2) < detection level[d]	2	10	–	
> detection level[d]	18	90	8010.22 ± 10193.1	
	VL among HIV-infected patients who received treatment (n = 4)			
	Min-Max		Mean ± SD	Test of significance (p value)
VL (1)	15400 – 27000		19570 ± 5366.40	
VL (2)	12000 – 34000		21450 ± 9187.49	
	VL among HIV-infected patients who did not receive treatment (n = 16)			
	No.	%	Mean ± SD	Test of significance (p value)
VL (1) < detection level[d]	2	12.5	–	$Z^{L} = -3.18$ (0.00)**
> detection level[d]	14	87.5	3396.14 ± 5227.32	
VL (2) < detection level[d]	2	12.5	–	
> detection level[d]	14	87.5	4170.29 ± 6703.13	

Abbreviations:
HIV – human immune deficiency virus; VL – viral load; Z – Z value of Wilcoxon Signed Ranks test;
** – significant at $p \leq 0.01$; VL (1) – VL measured at the beginning of the study;
VL (2) – VL measured after one year of the study; [d] – detection level was 50 IU/ml;
[&] – VL (1) – VL (2) for HIV patients whose viral load was above detection level (n = 18);
[L] – VL (1) – VL (2) for HIV patients whose viral load was above detection level (n = 14)

HIV (68.3%) compared with employed group (46.7%) ($X^2 = 6.83$; $p = 0.00$). Moreover, 34.4% of employed HIV-infected patients were PWID compared with 8.3% of unemployed group ($X^2 = 13.45$; $p = 0.00$). According to HIV progression, 17.8%, 63.3%, 4.4%, and 14.4% of employed HIV-infected patients had progressive, RP, LTNP, and controllers respectively, compared with unemployed patients (35%, 45%, 10% and 10%, respectively), ($^{\text{Monte Carlo}}$ $p = 0.03$) (Table IV).

Predictors of employment in the studied HIV-infected patients (n = 150) (Table V). Characteristics that were significantly associated with respect to employment status in univariate analysis were included in multivariate analysis using logistic regression model. The overall model was significant (model $X^2 = 57.11$, $p = 0.00$); significant predictors of employment were male gender (odds ratio [OR]: 7.29; 95% confidence interval [CI]: 2.72–19.52), and obtaining vocational training (OR: 6.04; 95%CI: 1.81–20.16). According to the results, HIV-infected man was 7.3 times more likely to be employed compared with HIV-infected woman. Moreover, HIV-infected patient who obtained vocational training was 6 times more likely to be employed than an illiterate HIV-infected patient. Although, in univariate analysis, having a family member living with HIV, PWID and HIV progression was significantly associated with employment; yet, these associations were no longer significant after adjustment for other covariates in multivariate analysis (Table V).

Discussion

The world has committed to ending the AIDS epidemic by 2030 as declared by UNAIDS 2016. The 90-90-90 target provides that by 2020: (a) 90% of all PLHIV will know their HIV status; (b) 90% of all people with diagnosed HIV infection will receive sustained antiretroviral therapy; and (c) 90% of people receiving antiretroviral therapy will achieve viral suppression (Global gains made towards the 90-90-90 targets UNAIDS, 2016)

In the present study males accounted for two thirds of cases. Similarly, 83% of HIV infected patients

Table IV
Characteristics of the studied HIV-infected patients with respect to patients' employment status (n = 150)

Characteristics	Employed HIV patients (n = 90)		Unemployed HIV patients (n = 60)		Test of significance (p value)
	No	(%)	No	(%)	
Gender					
Male	77	(85.6)	23	(38.3)	$X^2 = 36.12\ (0.00)^{**}$
Female	13	(14.4)	37	(61.7)	
Age (Years) (Mean ± SD)	35 ± 9.18		32.32 ± 9.18		t = 1.76 (0.08)
Marital status					
Single	41	(45.6)	19	(31.7)	$^{MC}p = 0.12$
Married	31	(34.4)	27	(45)	
Divorced	15	(16.7)	8	(13.3)	
Widowed	3	(3.3)	6	(10)	
Residence					
Rural	7	(7.8)	1	(1.7)	$^{FE}p = 0.14$
Urban	83	(92.2)	59	(98.3)	
Education					
Illiterate	19	(21.1)	19	(31.7)	$^{MC}p = 0.00^{**}$
Read & write	12	(13.3)	10	(16.7)	
Primary	6	(6.7)	10	(16.7)	
Secondary	10	(11.1)	11	(18.3)	
University	6	(6.7)	3	(5)	
Vocational training	37	(41.1)	7	(11.7)	
Family member living with HIV	42	(46.7)	41	(68.3)	$X^2 = 6.83\ (0.00)^{**}$
IV drug abuse	31	(34.4)	5	(8.3)	$X^2 = 13.45\ (0.00)^{**}$
Duration since HIV diagnosis					
< 5 years	33	(36.7)	25	(41.7)	$X^2 = -0.41\ (0.81)$
5 – 15 years	48	(53.3)	30	(50)	
> 15 years	9	(10)	5	(8.3)	
Co-morbidity$^{<}$	31	(34.4)	14	(23.3)	$X^2 = 2.11\ (0.14)$
Symptomatic HIV	71	(78.9)	50	(83.3)	$X^2 = 0.45\ (0.50)$
Type of progression					
Progressive (P)	16	(17.8)	21	(35)	$^{MC}p = 0.03^{*}$
Rapidly progressive (RP)	57	(63.3)	27	(45)	
Long-term non-progressive	4	(4.4)	6	(10)	
Controller	13	(14.4)	6	(10)	
CD4+ T cells (cells/mm^3)^					
< 100	7	(7.8)	8	(13.3)	$^{MC}p = 0.13$
100–199	3	(3.3)	6	(10)	
200–349	21	(23.3)	8	(13.3)	
≥ 350	59	(65.6)	38	(63.3)	
Viral load (IU/ml)$^{\$}$	(n = 11)		(n = 9)		$^{FE}p = 1$
< 10000	8	(72.7)	6	(66.7)	
≥ 10000	3	(27.3)	3	(33.3)	
Receiving cART	73	(81.1)	48	(80)	$X^2 = 0.02\ (0.86)$

Abbreviations:
HIV – human immune deficiency virus; st. – status; $^{<}$ – diabetes mellitus, hypertension, hepatitis C virus, hepatitis B virus; cART-combined antiretroviral therapy; ^ – baseline CD4 T-helper counts; X^2 – Chi square test; FE – Fisher's Exact test; MC – Monte Carlo test; t – student t-test; SD – standard deviation; * – significant at $p \leq 0.05$ (2-tailed); ** – significant at $p \leq 0.01$ (2-tailed)

Table V
Predictors of employment in the studied HIV-infected patients (n = 150)[a]

Characteristics	Multivariate analysis (logistic regression)			
	β	OR[#]	95%CI	p value
Gender				
Female^	–	–	–	–
Male	1.98	7.29	2.72–19.52	0.00**
Education				
Illiterate^	–	–	–	–
Read and write	0.60	1.82	0.50–6.58	0.35
Primary	–0.19	0.82	0.20–3.33	0.78
Secondary	0.07	1.07	0.30–3.85	0.91
University	0.94	2.57	0.42–15.57	0.30
Vocational training	1.79	6.04	1.81–20.16	0.00**
Having family member living with HIV				
Yes	0.16	1.17	0.45–3.07	0.74
No^	–	–	–	–
IV drug abuse				
Yes	0.93	2.54	0.78–8.28	0.12
No^	–	–	–	–
Disease progression				
Progressive	–1.23	0.29	0.06–1.22	0.09
Rapidly progressive	–0.515	0.59	0.16–2.22	0.44
LTNP	–1.09	0.33	0.04–2.39	0.27
Controller^	–	–	–	–
Model X^2 = 57.11 (0.00)**				

Abbreviations:
HIV – human immune deficiency virus; LTNP – long term non-progressive; IV – intravenous;
[a] – Multivariate odds ratios using logistic regression; ^ – Reference;
[#] – Odds ratios adjusted for all variables listed in the table; X^2 – Chi square; ** – significant at $p \leq 0.001$

reported were males with same age range in an Egyptian survey (Jackobsen, 2014).

The majority of our patients had urban residence (94.7%) and Amrya district showed the highest clustering (30.7%). Amrya district was also found to be the second highest district of HCV clustering in a PhD study conducted at the AFM (Gamaleldeen, 2016).

Sexual transmission and drug abuse remain the major routes of HIV infection in MENA including Egypt (Gokengin *et al.*, 2016). Our results of HIV due to IV abuse is in harmony with the results of a national surveillance study showing that 28% of reported HIV infected patients in Egypt in 2010 occurred in PWID which is consistent with an epidemic that disproportionately affects MSM and/or PWID (NAP, HIV/AIDS biological and behavioral surveillance; 2010). Male PWID comes out to 93,314; 0.37% of the male population aged 18–59 (Jackobsen, 2014).

In the last few years, many countries in MENA have been affected by social and political unrest and conflict. However, the current HIV prevalence of 0.1% is still among the lowest rates globally (El Beih *et al.*, 2012). Iran has the highest numbers of PWID (185 000, 0.43%), followed by Pakistan (117 000) and Egypt (93 000) (Mumtaz *et al.*, 2014).

HCV is a common co-infection among PLHIV in Egypt. This is due to the high background of HCV prevalence in the general population (7%) (EHIS, 2015). As in most countries, Egypt is adopting nucleic acid testing in blood banks to provide safe blood transfusions (Egyptian national standards for blood transfusion, 2011).

The development of signs and symptoms of AIDS typically parallels laboratory testing for CD4 lymphocytes. CD4 lymphocyte count, HIV VL and follow up duration are key components used to define HIV phenotypes (Gurdasani *et al.*, 2014).

In our study group, RP accounted for 56% while average percent is around 10–15% (Gurdasani *et al.*, 2014). This could be attributed to that those patients have signs and symptoms so they are more keen to show up for treatment and follow up. Also, PLHIV is

still a stigma as described in the Egyptian society for population studies (Stigma experienced by people living with HIV in Egypt, 2013).

In the current study, LTNP accounted for 6.7% which is similar to average estimates (5–10%) (Gurdasani *et al.*, 2014). EC proportion varies greatly between studies, it ranged from 0.15–7.70% according to the Concerted Action on SeroConversion on AIDS and Death in Europe (CASCADE) dataset as it did not necessarily reflect the length of follow-up required by the definition.

The best laboratory measure for determination of the long term progression of AIDS for therapeutic purposes is the level of HIV RNA in peripheral blood. Despite ART, the reconstitution of the immune system may be partial or incomplete, with considerable variability in the magnitude of the response (Gunthard *et al.*, 2016). CD4 count response is controversial, it mostly increases but still may remain below normal.

In our study, the significantly higher CD4+(4) count among the whole cohort and among the treated group as well as significantly higher VL (2) among the untreated group intensify the importance for ART in HIV patients.

CCR5 is a prominent cofactor for HIV-1 entry. 74 mutations including the intensively studied 32 base pair deletion (CCR5-delta32) were identified. We detected no deletion mutations among selected LTNP cases. Mutation occurs most frequently in the Caucasian population, while it cannot be found in the Asian, Middle East, African, and the American Indian population (Gero *et al.*, 2011).

In the present study, the unemployment rate among HIV-infected patients was 40%. The result coincides with unemployment rates of 45–65% reported by Dray-Spira *et al.* (2007) and 40% reported by Rabkin *et al.* (2004). However, it is much higher than unemployment rate; 12.1%, among HIV-infected patients reported in Gros *et al* (2016) which could be attributed to national difference; the overall unemployment rate in Rabkin *et al.* (2004) in Germany (4.6%) is lower than that in Egypt according to ILO, 2016 (12.7%). Yet, results of the current research are consistent with recent studies that indicates higher unemployment rates of HIV-infected individuals compared with the background population.

On studying predictors of employment for HIV-infected patients, HIV-infected men were more likely to be employed than HIV-infected women. Likewise, Dray Spira *et al.* (2007), reported lower rates of employment among HIV-infected women (46.9%) compared with HIV-infected men (65.1%), however, in the current study, employment rate among women was very much lower than Dray Spira *et al.*, 2007 (14.4% *vs* 46.9%). This could be partially due to the fact that women, even in absence of HIV disease, have lower opportunity regarding labor market participation especially in developing countries. In the general population in Egypt, unemployment among females is 25.7%, compared with 8.9% in males (CAPMAS, 2016).

Furthermore, in the present research, HIV-infected patients who obtained vocational training were more likely to be employed compared with illiterate HIV-infected patients. Similarly, previous studies showed higher probability of labor market participation in highly educated HIV-infected individuals as described by Elzi (2016) and that poor education is significantly associated with higher unemployment rate among HIV-infected patients (Gros *et al.*, 2016).

According to HIV/AIDS Strategic Frame 2012–2016; Egypt's goal is to stabilize the growth of the AIDS epidemic in the country, prevent new infections especially within the most at risk population and improve health outcomes for PLHIV. National positive response developments; most notably prevention programmes for the key populations of PWID and MSM, have been implemented. HIV testing is available for diagnostic purposes and both governmental and nongovernmental voluntary testing and counselling are being expanded. Treatment centers have been set up at fever hospitals. Stigma and discrimination are prohibited and various care and supportive measures are undertaken.

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SHORT COMMUNICATION

Analyses of Plasmids Harbouring Quinolone Resistance Determinants in *Enterobacteriaceae* Members

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Abstract

The aim of this study was to explore the plasmid characteristics of eight clinical *Enterobacteriaceae* strains containing extended broad spectrum beta-lactamases and plasmid-mediated quinolone resistance. Plasmids were transferred by conjugation or transformation and resistance determinants were investigated by PCR. We showed that at least one plasmid harbouring *qnr*B or *qnr*S determinant was transferred by conjugation in five isolates. *Qep*A determinant was confirmed to be on a non-conjugative plasmid. We found at least one beta-lactamase gene in seven of the eight clinical isolates having plasmid-mediated quinolone resistance, which indicated that these two resistance determinants were mostly on the same conjugative plasmids.

Key words: beta-lactamase, ESBL-producing *Enterobacteriaceae*, plasmid-mediated quinolone resistance

There has been increasing rate of resistance to quinolones and beta-lactam group of agents in *Enterobacteriaceae* members recently. The most frequently seen resistance mechanism against beta-lactam antibiotics is the production of extended spectrum beta-lactamase (ESBL) and the most common ESBLs are the plasmid-borne CTX-M, SHV, and TEM types of enzymes (Pfeifer *et al.*, 2010). Studies have reported that most of the ESBL-producing *Enterobacteriaceae* members are also resistant to fluoroquinolones (Nazik *et al.*, 2011; Pasom *et al.*, 2013; Kim *et al.*, 2014). The role of mutations occurring in the regions encoding DNA gyrase and topoisomerase IV enzymes and overexpression of efflux pumps in the resistance to fluoroquinolones have been known for a long time. The development of plasmid-borne resistance to quinolones in *Enterobacteriaceae* members was first reported in a *Klebsiella pneumoniae* isolate. The Qnr protein, causes a low level quinolone resistance by binding to bacterial DNA gyrase and topoisomerase IV. The *qnr*A gene that had been seen in many *Enterobacteriaceae* members worldwide was followed by *qnr*B, *qnr*C, *qnr*D, *qnr*S and *qnr*VC genes. The other more recent plasmid-borne resistance mechanisms include: *qep*A and *oqx*AB, which are efflux pump encoding genes, and *aac(6')-Ib-cr*, which contributes to the resistance by modifying ciprofloxacin (Pasom *et al.*, 2013; Jacoby *et al.*, 2014). The aim of this study was to explore various plasmid characteristics of ESBL-positive *Escherichia coli* and *K. pneumoniae*, which have been found to have plasmid-mediated quinolone resistance.

The present study included four *E. coli* and four *K. pneumoniae* isolates, which were isolated from various clinical specimens at the Medical Microbiology Department of Ege University Hospital. All the isolates were proved previously to be ESBL-positive by a double disk synergy test and to have at least one plasmid-mediated quinolone resistance (PMQR) determinant (Hoşgör-Limoncu *et al.*, 2012). Minimum inhibitory concentration (MIC) values of ciprofloxacin were determined using the microdilution method as suggested by the Clinical and Laboratory Standards Institute (CLSI). *E. coli* ATCC 25922 was used as the control strain.

Plasmids were isolated from the clinical bacterial isolates and transconjugants by a commercial kit (QIAGEN plasmid maxi kit, Germany) according to the recommendations of the manufacturer. The *qnr*A, *qnr*B, *qnr*S, and *qep*A genes were explored in the isolated plasmids with the PCR method using original primers (Gay *et al.*, 2006; Yamane *et al.*, 2008; Minarini *et al.*, 2008).

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The positive control strains and plasmids used for *qnr*A, *qnr*B, *qnr*S, and *qep*A were *E. coli* j53 (pMG252), *E. coli* j53 (pMG298), *E. coli* J53 (pMG306) and pSTV28qepA, respectively (Hoşgör-Limoncu *et al.*, 2012). 0.75% and 1.5% agarose were prepared with 1 × TBE for plasmid DNA and PCR products, respectively.

The PMQR gene transfer was performed by the broth culture mating method using *E. coli* J53 Azi^R as a recipient (Jacoby and Han, 1996). From the suspensions of 0.5 McFarland turbidity, first 0.1 ml of each donor strain and then 1 ml of recipient strain (*E. coli* J53 Azi^R) were inoculated into falcon tubes containing 4.5 ml of Luria-Bertani broth (LB-Becton, Dickinson). The tubes were incubated in a 35°C water bath for 18 hours. Transconjugants were selected on LB plates containing sodium azide (100 mg/l) (Sigma-Aldrich, Germany) and nalidixic acid (6 mg/l) (Sigma-Aldrich, Italy). The *qnr*A, *qnr*B, *qnr*S, and *qep*A genes were explored in the plasmids that were isolated from transconjugants with the PCR method using original primers (Gay *et al.*, 2006; Yamane *et al.*, 2008; Minarini *et al.*, 2008).

The transformation of qnr plasmids to *E. coli* DH5α cells was performed by heat shock (Sambrook *et al.*, 1989). Competent *E. coli* DH5α suspension (200 µl) was transferred to sterile microcentrifuge tubes, and plasmid DNA (10 µl– 50 ng) was added to these tubes. The mixtures in the tubes were kept on ice for 30 min by stirring, and then they were kept 90 sec in a water bath adjusted to 42°C. At the end of the incubation they were quickly put on ice and kept there for 1–2 min. Finally, 800 µl of LB medium was added and they were kept for 45 min in a shaker incubator (250 rpm). The transformants were selected on LB agar plates containing ciprofloxacin (0.025–1 µg/ml), and their DNA was isolated to identify the relevant genes by PCR.

The presence of TEM, SHV and CTX-M beta-lactamase genes was explored by PCR in the clinical isolates with identified PMQR determinants and in their transconjugants-transformants (Taşlı and Bahar, 2005, 2010).

Plasmids were isolated from the clinical strains harbouring PMQR determinants. These isolates were found to contain plasmids ranging from approximately 1.5 kbp to 40 kbp. Conjugation experiments were carried out to explore the transferability of plasmids having PMQR determinants. It was observed that ~23 and ~40 kbp plasmids of isolate 130 were transferred. From the three plasmids of isolate 151, only the one of ~23 kbp was transferred; and from the two plasmids of isolate 160, only the one of ~23 kbp was transferred. From the two plasmids of isolates 134 and 146, only those plasmids of ~23 kbp were transferred. The plasmids of ~20 kbp of isolates 13 and 73 and the plasmid of ~23 kbp of isolate 140 could not be transferred to the recipient bacteria by conjugation.

The presence of PMQR determinants in the transconjugants was explored by PCR. It was verified that *qnr*B (in the ~23 kbp plasmid) in isolates 130 and 160, also *qnr*S (in the ~23 kbp plasmid) in isolate 151 were transferred. It was also verified with PCR that *qnr*B was transferred in isolates 134 and 146, but no *qnr*A determinant could be detected in their conjugants (Fig. 1). Therefore, the ~23 kbp conjugative plasmids of isolates 134 and 146 were revealed to carry *qnr*B determinants but not *qnr*A. Transformation experiments were performed with the plasmids of these isolates, but no results could be obtained.

While the transformation trials failed for the plasmids of isolates 13 and 140, it was turned out successful for the plasmid of isolate 73. The presence of *qep*A determinant was verified through the PCR made from the transformant of this strain (Fig. 1). The ciprofloxacin MIC values of the transformant of isolate 73 and recipient strain were 0.2 µg/ml and 0.025 µg/ml, respectively. The ciprofloxacin MIC value was increased 8-fold in the transconjugate.

The presence of TEM, SHV and CTX-M beta-lactamase genes was explored by PCR in the eight clinical isolates harbouring PMQR determinants. TEM, SHV and CTX-M were seen together in three of the iso-

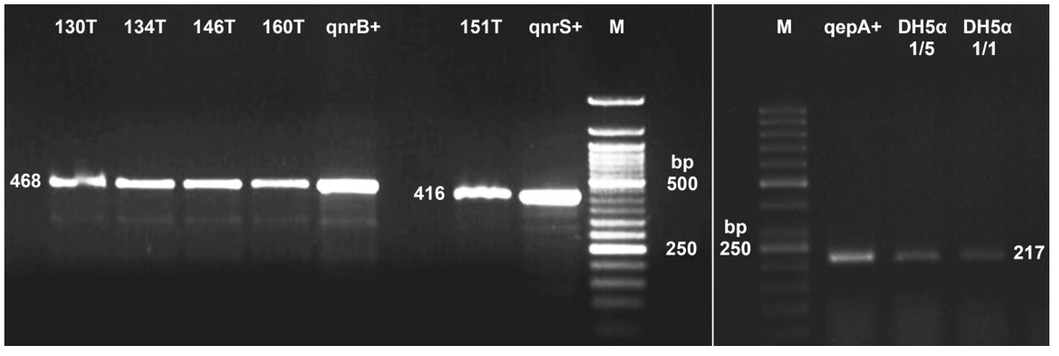

Fig. 1A. PCR results of *qnr*B and *qnr*S from transconjugants of the isolates and Fig. 1B. PCR result of *qep*A from transformant of isolate 73.

Table I
Plasmids and beta-lactamase types of PMQR-positive clinical isolates and transconjugants (*Transformant)

Isolate Number/ Species	PMQR determinant	Plasmid count		Beta-lactamase type	
		Original strain	Transconjugant	Original strain	Transconjugant
13 *K. pneumoniae*	*qnr*S	1	–	TEM SHV	–
73 *E. coli*	*qep*A	1	1*	TEM CTX-M	TEM* CTX-M*
130 *K. pneumoniae*	*qnr*B	2	2	TEM SHV CTX-M	TEM CTX-M
134 *K. pneumoniae*	*qnr*A *qnr*B	2	1	TEM SHV CTX-M	TEM CTX-M
140 *E. coli*	*qnr*B	1	–	TEM CTX-M	–
146 *K. pneumoniae*	*qnr*A *qnr*B	2	1	TEM SHV CTX-M	TEM CTX-M
151 *E. coli*	*qnr*S	3	1	CTX-M	–
160 *E. coli*	*qnr*B	2	1	–	–

lates, TEM and CTX-M in two of them, and TEM and SHV in one. Only CTX-M was present in one isolate and none of these beta-lactamase determinants were encountered in another isolate (Table I). Transferability of beta-lactamase genes by conjugation or transformation was explored by PCR in the isolates (73, 130, 134 and 146). TEM and CTX-M beta-lactamase genes were found in the transformant of isolate 73 and in the transconjugants of isolates 130, 134 and 146. The SHV gene could not be detected in any of the examined transconjugants and transformants (Fig. 2).

Quinolones and beta-lactams are broad-spectrum antibiotics that are frequently used in both community and hospital-acquired infections. Due to plasmids play an important role in the development of resistance to these two antibiotic groups, it is important to investigate these plasmids in detail (Kanamori *et al.*, 2011, Jacoby *et al.*, 2014; Zhao *et al.*, 2015; Piekarska *et al.*, 2015). In our study, we detected plasmids sized about 1.5–40 kbp in clinical isolates with established PMQR determinants. We found that at least one plasmid (~ 23 kbp) was transferred by conjugation in five of the eight clinical isolates included in our study. The presence of *qnr*B and *qnr*S in these conjugative plasmids was verified with PCR (Fig. 1), but no *qnr*A could be detected. Transformation was successful in one of the three isolates whose plasmids could not be transferred through conjugation. The presence of *qep*A in this isolate's transformant was confirmed with PCR (Fig. 1). The ciprofloxacin MIC values of the transformant of isolate 73 and recipient strain were 0.2 µg/ml and 0.025 µg/ml, respectively. The ciprofloxacin MIC value was increased 8-fold in the transconjugate.

In a study conducted in Turkey with ESBL-positive 61 *E. coli* isolates, PMQR was found in only four isolates, and from these only *qnr*A and *qnr*S could be transferred by conjugation but not *qep*A. The ciprofloxacin MIC values have been reported to increase 8 to 62-fold in transconjugates (Nazik *et al.*, 2011). In a study made in Argentina, *qnr*B and *aac(6')-Ib-cr* were transferred through conjugation but the *qep*A determinant could be transferred by electro-transformation (Rincón *et al.*, 2014). While they reported that *qep*A was on a plasmid of around 97 kbp, in our study we found a plasmid of around 20 kbp in the strain which *qep*A was detected. The increase they found in the ciprofloxacin MIC value of the transformant was also the same as found in our study. Very different conjugation rates of plasmids harbouring PMQR determinants may be encountered among studies, such as 17% and 40% (Cai *et al.*, 2011; Pasom *et al.*, 2013). The rate of success in the transfer of plasmids through conjugation is influenced by the

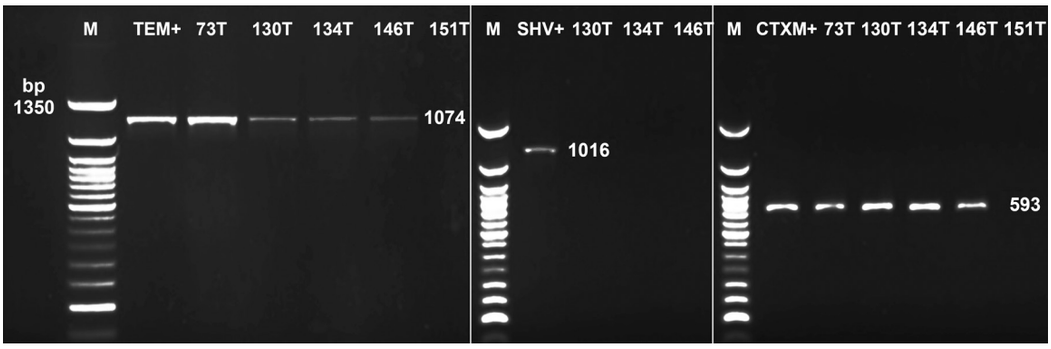

Fig. 2A. Presence of bla_{TEM}, bla_{SHV} (Fig. 2B) and bla_{CTX-M} (Fig. 2C) genes in transconjugants and transformants.

method used (*e.g.* "Filter mating"), recipient strains or antibiotics of varying effectiveness. Having carried out conjugation in five out of the eight clinical isolates in the present study, indicates a high rate of success. Similar to the results of some studies in the literature (Nazik *et al.*, 2011; Rincón *et al.*, 2014), we also found that the plasmid containing *qep*A is non-conjugative. However, Kim *et al.* (2014) stated that the *qep*A determinant could be transferred through conjugation from all of the four isolates containing this determinant.

The presence of TEM, SHV and CTX-M beta-lactamase genes was explored by PCR in the eight clinical isolates harbouring PMQR determinants. TEM, SHV and CTX-M were seen together in three of the isolates. Only CTX-M was present in one isolate and none of these beta-lactamase determinants were encountered in another isolate (Table I). Transferability of beta-lactamase genes by conjugation or transformation was explored by PCR in the isolates (73, 130, 134 and 146). TEM and CTX-M beta-lactamase genes were found in the transformant of isolate 73 and in the transconjugants of isolates 130, 134 and 146. The SHV gene could not be detected in any of the examined transconjugants or transformants (Fig. 2).

As a result, we found at least one beta-lactamase gene in seven out of the eight clinical isolates containing PMQR determinants. We determined that the PMQR and beta-lactamase genes were on the same plasmid in three out of the four isolates harbouring beta-lactamases and conjugative plasmids. The most frequently transferred beta-lactamase genes were bla_{TEM} and $bla_{\text{CTX-M}}$. We also found that the *qep*A determinant was transferred together with bla_{TEM} and $bla_{\text{CTX-M}}$. In our study, *qnr*B was found to be the most frequently transferred PMQR determinant.

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SHORT COMMUNICATION

Inhibition of Multidrug-Resistant Gram-Positive and Gram-Negative Bacteria by a Photoactivated Porphyrin

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Abstract

The authors studied the *in vitro* antibacterial activity of the photo-activated porphyrin meso-tri(N-methyl-pyridyl), mono(N-tetradecyl-pyridyl)porphine (C14) against four multidrug-resistant bacteria: *Staphylococcus aureus*, *Enterococcus faecalis* (Gram-positive), *Escherichia coli*, *Pseudomonas aeruginosa* (Gram-negative). Using 10 μg/ml of porphyrin and 60 sec irradiation we observed the remarkable susceptibility of *S. aureus* and *E. faecalis* to treatment while, under the same conditions, *E. coli* and *P. aeruginosa* showed very low susceptibility. In a later stage, suspensions of Gram-negative bacteria were processed with EDTA before photo-activation, obtaining a significant decrease in viable counts. In view of the results, if the combination of low porphyrin concentrations and short irradiation times will be effective *in vivo* also, this approach could be a possible alternative to antibiotics, in particular against localized infections due to multidrug-resistant microorganisms.

Key words: antibiotic-resistant bacteria; laser light irradiation; porphyrin; photo-activation treatment

Antibiotic resistance is a very current topic health concern and represents one of the most important challenges of the 21st century to human health because, due to extensive use over the last decades, antibiotics are gradually losing their effectiveness. For example, methicillin resistant *Staphylococcus aureus* (MRSA) isolated since the early 1960s, still represents more than 60% of all the *S. aureus* isolates in US hospitals (NNIS 2004), despite recent data demonstrating that the incidence of serious infections due to MRSA has decreased since 2005 in numerous settings (Dantes *et al.*, 2013). At the same time the appearance of a further resistance against vancomycin (vancomycin resistance *Staphylococcus aureus*, VRSA) (Sievert *et al.*, 2002) and its possible transfer by conjugation (de Niederhausern *et al.*, 2011), could aggravate the situation. Also *Enterococcus* spp., isolated from hospital and food-animal samples, has developed resistance against many antibiotics, including vancomycin (de Niederhausern *et al.,* 2007). Finally, considering the increase of multiresistant Gram-negative bacilli (MRGNB) such as *Klebsiella* spp., *Escherichia coli* and *Pseudomonas aeruginosa* to beta-lactam antibiotics (penicillins, cefalosporins, monobactams, carbapenems) (Shaikh *et al.*, 2015), it becomes increasingly urgent to experiment different antimicrobial treatments. A possible alternative is represented by photodynamic therapy (PDT), a treatment that achieves cytotoxic activity using a combination of visible light, a chemical compound photosensitizer and oxygen. The antimicrobial PDT was overtaken by the discovery of antibiotics, but today could offer new therapeutic opportunities for localized infections and those that don't require systemic therapies, especially if caused by multidrug-resistant bacteria. Among the several advantages of antimicrobial PDT, the most important are non-target specificity and the few side effects. Furthermore, bacterial inactivation is obtained with an action not related to the antibiotic-resistance mechanisms (Jori *et al.*, 2006; Tavares *et al.,* 2010). A remarkable variety of photosensitizing compounds (porphyrins, metallo-porphyrins and derivatives), when activated, have shown efficacy in the photo-killing of pathogenic bacteria regardless of their sensitivity or resistance to antibiotics (Lazzeri *et al.*, 2004; Merchat *et al.*, 1996b), but their efficacy can significantly change, relatively to the microorganism target (Huang *et al.,* 2010). Generally neutral and anionic photosensitizers exhibit considerable phototoxic activity against Gram-positive and

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no significant activity against Gram-negative bacteria, unless the outer membrane permeability is enhanced, prior to irradiation, by treatment with chelating agents (Reddi *et al.*, 2002). In contrast, cationic porphyrins, under appropriate conditions, promote efficient inactivation of Gram-negative bacteria also, without the need for modifying the permeability of the outer membrane (Merchat *et al.*, 1996a). During the planning of the PDT it must therefore take into account this particular feature, relative to the photosensitizers employed, as well as the operating conditions. It is therefore extremely crucial to define a protocol that would allow to obtain a reduction of the microbial cells at the same time preventing damage to the host tissues. In view of these premises, the present work investigated the antibacterial activity of a cationic porphyrin against two Gram-positive and two Gram-negative multidrug-resistant bacteria employing low concentrations of the photosensitizer, short incubation in the dark and short times of exposure to a monochromatic laser light. In a second step the Gram-negative bacteria were subjected to the same treatment after exposure to EDTA.

The following microorganisms, all from the American Type Culture Collection (Manassas, VA, USA), were used: methicillin resistant *S. aureus* ATCC BAA-2094 and vancomycin resistant *E. faecalis* ATCC BAA-2128 (Gram-positive), multidrug-resistant *E. coli* ATCC BAA-2452 and *P. aeruginosa* ATCC BAA-2109 (Gram-negative). The strains were grown at 37°C for 24 h in Tryptic Soy broth or Tryptic Soy agar (TSB or TSA, Difco Laboratories, Detroit, MI) and were maintained at 80°C in the appropriate cultivation broth containing 20% (v/v) glycerol (Merck, Darmstadt, Germany).

We used a meso substituted tetracationic porphyrine (meso-tri(N-methyl-pyridyl), mono(N-tetradecyl-pyridyl)porphine (C14) (kindly provided by Prof. Jori, European Patent Application EP 1 457 113 A1 2004) a synthetic compound of the tetrapyrrole series, having three positive charges situated in peripheral substituents of the tetrapyrrolic macrocycle core (alkyl chain), and one hydrophobic hydrocarbon chain of 14 carbon atoms as a peripheral substituent tail. Cell irradiation experiments were performed by using a diode laser with 635 nm wavelength, output power 50 mW, 300 nm diameter optical fibers (LAMBDA Scientifica, Vicenza, Italy).

Overnight cultures in TSB of *S. aureus* ATCC BAA-2094, *E. faecalis* ATCC BAA-2128, *E. coli* ATCC BAA-2452 and *P. aeruginosa* ATCC BAA-2109, were centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was discarded and the cells were harvested, washed twice, and resuspended in Phosphate Buffer Saline (PBS, pH 6) to give of about $l0^7$ CFU/ml. The resultant bacterial suspensions were aseptically distributed in eppendorf tubes and added of suitable volume of porphyrin C14 to a final concentration of 5 and 10 μg/ml. The bacterial cells were mixed carefully, incubated in the dark and room temperature for 60", to allow the binding and/or uptake of the porphyrin, and subjected to irradiation for 30"and 60" using a monochromatic light laser. The laser fibre was placed in the bottom of eppendorf tube and a spiral movement was manually performed to ensure uniform diffusion of the light. Afterward tenfold dilutions of each suspension were seeded on TSA and, following a 24 h incubation at 37°C, viable counts (CFU/ml) were determined. Two controls were performed (only laser light irradiation and porphyrin without any irradiation).

In a second step of the study, in consideration of the low susceptibility of Gram-negative bacteria to the PD treatment, suspensions of *E. coli* and *P. aeruginosa* were processed with EDTA (3 mM) for 60" before the treatment with 10 μg/ml of porphyrin and 60" of laser light irradiation.

The experiments were repeated three times, the data (log bacterial count) were averaged and standard deviation was calculated. Bacterial reduction in percentage was determined using the following formula:

$$R\% = [(B-A) / B] \times 100$$

R: percentage reduction of the microbial cells;
A: sample microbial suspension (CFU/ml);
B: control microbial suspension (CFU/ml)

The decline rates of *S. aureus*, *E. faecalis*, *E. coli* and *P. aeruginosa* were analyzed using a t-test for paired data. A statistical probability equal to or less than 0.05 was considered significant.

The antibacterial activity of the photo-activated porphyrin against *S. aureus* ATCC BAA-2094, *E. faecalis* ATCC BAA-2128, *E. coli* ATCC BAA-2452 and *P. aeruginosa* ATCC BAA-2109 is summarized in Table I. The Gram-positive bacteria showed a remarkable susceptibility to photodynamic treatment. With the highest parameters employed (10 μg/ml of porphyrin, 60" of dark incubation time and 60" of irradiation) we obtained a bactericidal activity with viable counts decrease of 3.7 and 4.4 log CFU/ml for *S. aureus* and *E. faecalis* (99.98% and 99.996% reduction, respectively) ($p < 0.01$ compared to controls). Even using the lower parameters (5 μg/ml of porphyrin, 60" of dark incubation time and 30" of irradiation) we obtained a significant reduction (99.29% and 99.52%, respectively). In the same experimental conditions, and also employing the highest treatment parameters, the Gram-negative bacteria *E. coli* and *P. aeruginosa* showed a lower susceptibility (decrease respectively of 40.8% and 73%, $p > 0.05$). This outcome is probably due to the low outer membrane permeability that affects the uptake of the porphyrin (Reddi *et al.,* 2002). Our results against the Gram-negative bacteria, are partially in disagreement with the studies of Merchat *et al.* (1996a) who found

Table I
Antibacterial activity of photo-activated porphyrin against *S. aureus*, *E. faecalis*, *E. coli* and *P. aeruginosa* after 1 min dark incubation

Start	laser 30”	laser 60”	Por 5 μg	Por 10 μg	Por 5 μg laser 30”	Por 5 μg laser 60”	Por 10 μg laser 30”	Por 10 μg laser 60”
S. aureus	6.96* ± 0.07	6.95 ± 0.08	5.98 ± 0.04	5.53 ± 0.04	4.87 ± 0.01	4.28 ± 0.03	3.87 ± 0.01	3.25 ± 0.02
7.02 ± 0.04*	12.5%**	13.46%	90.76%	96.73%	99.29%	99.82%	99.93%	99.98%
E. faecalis	7.02 ± 0.08	7.11 ± 0.07	5.14 ± 0.02	4.95 ± 0.07	4.90 ± 0.03	4.56 ± 0.09	3.17 ± 0.05	2.83 ± 0.06
7.22 ± 0.05	37.12%	22.15%	91.79%	99.46%	99.52%	99.78%	99.991%	99.996%
E. coli	7.18 ± 0.04	7.06 ± 0.06	7.12 ± 0.04	7.11 ± 0.06	7.10 ± 0.08	7.08 ± 0.01	7.02 ± 0.03	6.99 ± 0.05
7.21 ± 0.02	8.7%	8.7%	20.12%	20.7%	21.95%	26.2%	35.5%	40.8%
P. aeruginosa	5.73 ± 0.09	5.74 ± 0.03	5.63 ± 0.03	5.56 ± 0.08	5.61 ± 0.05	5.60 ± 0.02	5.54 ± 0.07	5.51 ± 0.02
6.08 ± 0.06	55%	54%	64.4%	69.7%	65.67%	66.6%	70.83%	73%

* Log CFU/ml, ** % reduction, Por = porphyrin

that cationic porphyrins like C14 show bactericidal activity without the addition of chelating agents. This discrepancy could be due to the feature of our experimental design, carried out in mild operating conditions: low dark incubation time with photosensitizer (60”) and low laser light irradiation time (30”–60”). In the second step of the study, the addition of EDTA to the cell suspensions before PD treatment has produced a decrease of about 2,7 and 3,5 log CFU/ml in *E. coli* and *P. aeruginosa* viable counts (Table II), with a reduction of 99.83 and 99.97 %, respectively ($p < 0,01$). Relatively to controls, the only exposure to laser light does not caused significant effects on bacterial viability, while the not photo-activated porphyrin at the highest concentration (10 μg/ml) produced a reduction of 1.5 and 2.3 log CFU/ml for *S. aureus* and *E. faecalis*, and lower than 1 log for *E. coli* and *P. aeruginosa*. This antibacterial activity observed without laser light irradiation could be ascribed to the presence of the long hydrocarbon tail, which can interact with hydrophobic areas in the cell membrane, inducing a marked alteration of the native three-dimensional architecture and impairing specific metabolic processes (Maraggia, 2006).

According to our results if the experimental design employed in this study is proved effective in bacterial photo-inactivation also *in vivo*, the antimicrobial PDT could be suitably used for less invasive treatments that do not require systemic antibiotic therapies. Porphyrin C14 or a similar photosensitizer could be employed for the treatment of localized infections, chronic wounds, oral candidiasis and in the dental field for cariogenic and periodontal diseases (Jori *et al.*, 2006) in particular when caused by multidrug-resistant Gram-positive and Gram-negative bacteria. The low sensitivity of Gram-negative bacteria observed in our study can be overcome by employing a combination of porphyrin and EDTA. Using this association we achieved a reduction in viable counts such as those observed for the Gram-positive bacteria. The advantage of PDT, as appears from our data, is that the bactericidal activity is obtained employing low dark incubation time with photosensitizer, low dosages of porphyrin and very short irradiation times; as previously referred by Dai *et al.* (2009), if irradiation is performed at short intervals after photosensitizer application (minutes), the PDT damage to host tissue will be minimized. Membranes and cell wall components are the main targets of PDT and the photosensitizers do not need to enter the cell, but the adhesion to these structures is sufficient for bacterial inactivation. In this way the microorganisms don’t have the possibility to develop resistance through the known mechanisms. Unlike antibiotics, repeated

Table II
Antibacterial activity of photo-activated porphyrin against *E. coli* and *P. aeruginosa*
(60” dark incubation with 10 μg/ml of porphyrin) after EDTA addition

Start	Laser 60”	Por 10 μg	EDTA 60”	EDTA 60” Por 10 μg	Laser 60” Por 10 μg	EDTA 60” Laser 60” Por 10 μg
E. coli	7.6* ± 0.07	7.58 ± 0.04	7.54 ± 0.08	7.28 ± 0.02	7.34 ± 0.05	4.92 ± 0.02
7.70* ± 0.04	18%**	24%	30%	62%	56%	99.83%
P. aeruginosa	7.40 ± 0.03	7.34 ± 0.08	7.34 ± 0.09	7.32 ± 0.05	5.34 ± 0.04	4.38 ± 0.07
7.89 ± 0.04	67.53%	71.81%	71.81%	72.98%	99.72%	99.97%

* log CFU/ml, ** % reduction, Por = porphyrin

photodynamic treatments do not seem to induce the selection of resistant bacteria, as singlet oxygen and free radicals interact with numerous cell structures and different metabolic pathways of the microorganisms (Wainwright and Crossley, 2004). Consequently, this therapeutic approach may be a viable alternative to the use of antibiotics in particular against infections due to multidrug-resistant bacteria, opening new prospects for the use of photosensitized processes in the medical field.

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SHORT COMMUNICATION

Metagenomic Profiling of the Bacterial Community Changes from Koji to Mash Stage in the Brewing of Soy Sauce

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Abstract

The improvement of soy sauce fermentation is restricted by the insufficient information on bacterial community. In this study, bacterial communities in the koji and mash stage were compared based on next-generation sequencing technology. A total of 29 genera were identified in the koji stage, while 34 in the mash stage. After koji stage, 7 genera disappeared and 12 new genera appeared in the mash stage. The dominant bacteria were *Kurthia*, *Weissella* and *Staphylococcus* in the koji stage and *Staphylococcus*, *Kurthia*, *Enterococcus* and *Leuconostoc* in the mash stage. The results provided insights into the microbial communities involved in soy sauce fermentation.

Key words: metagenomics, microbial community, next-generation sequencing, soy sauce fermentation

Soy sauce, a traditional condiment popular in East Asia, is fermented with a mixture of soybean, wheat and bran (Tanasupawat *et al.*, 2002). Soy sauce manufacturing is based on natural inoculation and mixed fermentation under an open environment; thus, its microbial community structure is complex and has significant diversity. It is important to identify which microbes are essential for soy sauce brewing. However, only a few dominant microbes have been identified by the culture-dependent analysis method and the culture-independent method based on DGGE. A more comprehensive understanding of the efficient microbes in the different fermentation stages is necessary to improve soy sauce fermentation.

The fermentation of soy sauce involves two fermentation stages: koji fermentation and mash fermentation. It is the addition of brine that triggers the transition from the koji to the mash stage. Addition of brine is crucial to produce the typical flavour of soy sauce. Brine changes microbial growth conditions and therefore affects the microbial community. There were less fungal species in the microbial community, and their composition and changes are relatively clear. However, insufficient information was supplied on the differences in bacterial communities between the koji and mash stage, restricting the improvement of soy sauce production.

Most studies on the microbial components of soy sauce fermentation used cultivation methods (Ito and Dou, 1994; Tanaka *et al.*, 2012; Tanasupawat *et al.*, 2002). These methods do not have the capacity to reveal all microbial communities involved in soy sauce fermentation. Many microbes in the natural environment are non-cultivable by current isolation and culture methods (Mamlouk *et al.*, 2011; Torsvik and Øvreås, 2002). Metagenomics, which does not depend on microbial isolation and culture, can be used to investigate microbial community. Metagenomics has been widely used to study fermented food, activated sludge, water bacteria and rumen (Liaw *et al.*, 2010; Kakizaki *et al.*, 2012; Dai *et al.*, 2012). To date, no metagenomic study has focused on the microbial community of soy sauce.

Soy sauce koji and mash samples were collected from the soy sauce factory (30°04′ 01″N, 120°35′54″E) in Shaoxing. They were sampled at the end points of their fermentation stages. The soy sauce was fermented according to the method described by Röling with modifications (Röling *et al.*, 1996). Fermentation was carried out as follows. Defatted yellow soybean was boiled and then cooled to room temperature (RT, approximately 30°C), and wheat was roasted and then cooled to RT before being ground. A mixture of steamed defatted yellow soybean and ground wheat

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(V/V, 5.5:4.5) was inoculated with *Aspergillus oryzae* and kept at 35°C for 42 h to form soy sauce koji. Koji was mixed with 2.5 times the volume of salted water (18 Be' to 20° Be') in a fermentation tank and held at RT for 6 months to produce soy sauce mash.

Five parallel samples were collected from five batches of soy sauce fermentation at the end of the koji and mash stages respectively. The samples from the koji or mash stage were mixed for metagenomics analysis. DNA was isolated from the frozen samples within 3 weeks of collection. Metagenomic DNA was extracted by direct DNA extraction techniques following the method described by Ni (Ni *et al.*, 2010) . With the metagenomic DNA from the koji or mash stage as templates, V3 regions of the 16S rRNA gene were amplified using universal primers V3-F (5'-CTACGGGAGGCAGCAG-3') and V3-R (5'-ATTACCGCGGCTGCTGG-3') (Ovreås *et al.*, 1997). The amplified V3 regions of the 16S rRNA gene were purified using a DNA Purification Kit (Tiangen Biotechnology, China). The purified PCR products were sequenced by Beijing Genomics Institute (BGI) using HiSeq 2000 (Illumina, USA). Mothur software was used to analyse the sequences. Each sequence was assigned in comparison to sequences in the EzTaxon-extended database using BLASTN searches and pairwise similarity comparisons (Chun *et al.*, 2007). Bacterial species were identified following the approach described by Huse (Huse *et al.*, 2008). Sequences that showed more than 97% similarity were regarded as the same operational taxonomy unit (OTU) (Altschul *et al.*, 1997). Bacterial community structure was analysed using OTU-based approaches.

The purity and yield of metagenomic DNA extracted from the koji and mash were assessed according to absorbance ratios. The A_{260}/A_{280} and A_{260}/A_{230} values of the koji were 1.81 and 1.98, respectively, whereas those of the mash were 1.80 and 2.12, respectively. These results indicate ideal DNA purity. The yields of the koji and mash stages were 0.26 and 0.06 μg DNA/g, respectively, indicating that the biomass of the former

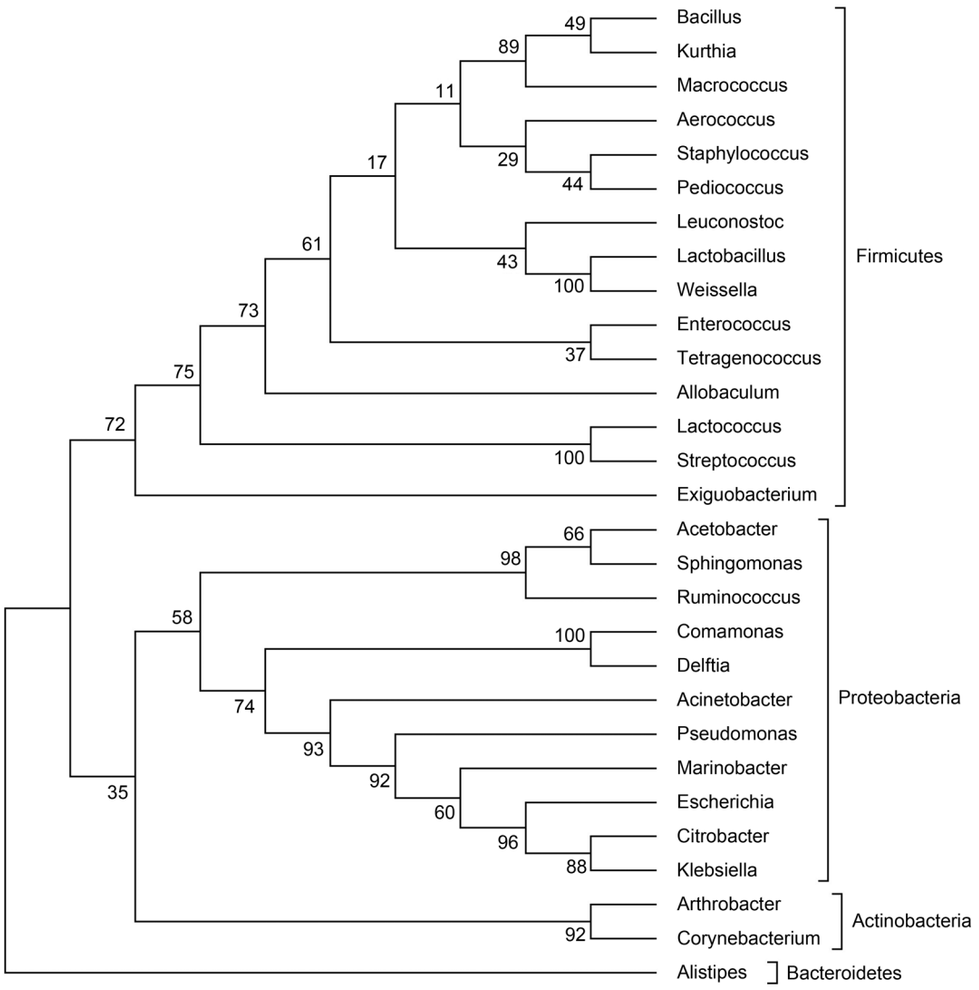

Fig. 1. Neighbor-joining tree of the identified 120 OTUs from the koji

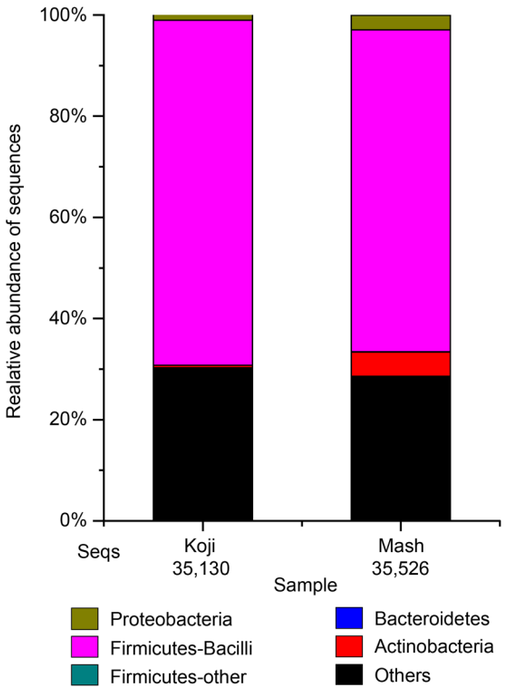

Fig. 2. Relative abundance of sequences from the koji and mash samples

was greater than that of the latter. As expected, intense bands of agarose gel electrophoresis of amplified V3 regions of 16S rDNA were approximately at 200 bp. Next-generation sequencing yielded 70,656 V3 region-trimmed sequences, of which 35,130 were from the koji and 35,526 were from the mash. Their lengths were distributed mainly at approximately 160 bp, and none was shorter than 106 bp or longer than 230 bp. The results were consistent with the known length of bacterial V3 regions (Whiteley *et al.*, 2012).

The OTUs generated by statistical analysis were used to identify species and generate a microbial diversity profile. A total of 181 and 224 OTUs were generated from the koji and mash, respectively. After matching with the database, 0.15% of the OTUs from the koji were mapped at the species level, 67.4% at the genus level and 32.4% were found undescribed in the database. Similarly, 0.22% of the OTUs from the mash were mapped at the species level, 62.1% at the genus level and 37.7% were found undescribed in the database. As shown in Fig. 1, most bacterial in the koji belonged to Firmicutes (103 OTUs, 23,929 sequences, 68.19% of the 35,130 sequences in total); the minority belonged to Proteobacteria (13 OTUs, 343 sequences, 1% of the total sequences), Actinobacteria and Bacteroidetes (4 OTUs, 101 sequences, 0.3% of the total sequences). Within Firmicutes, 101 OTUs (68.1% of the total sequences) belonged to bacilli. As shown in Fig. 2, the proportion of the V3 regions of 16S rRNA gene sequences belonging to bacilli (Firmicutes) were high for both koji (68.1% of the 35,130 sequences in total) and mash (63.42% of the 35,526 sequences in total). The results indicated that most bacteria in the mash came from the koji.

Genus-level identification for both koji and mash is shown in Fig. 3. The relative abundance of each genus in the entire bacterial community is also given. The relative abundance of each genus was calculated as the percentage of its matched sequences from the total sequences. Subsequently, 29 and 34 genera were identified in the koji and mash, respectively. Compared with those in the koji, 7 genera disappeared and 12 genera appeared in the mash. However, these genera only accounted for a very small percentage of the total abundance. The most common genera between the koji and mash were *Kurthia* and *Staphylococcus*, followed by *Weissella* and *Leuconostoc*. The relative abundances of *Acinetobacter*, *Corynebacterium*, *Lactococcus*, *Macrococcus* and *Streptococcus* increased when the fermentation entered the mash period. Some bacterial genera, such as *Streptomyces*, *Chryseobacterium*, *Paracoccus*, *Aquabacterium*, *Proteus*, *Providencia*, *Salmonella*, *Enhydrobacter*, *Aeromonas* and *Vibrio*, were only detected in the mash.

The microbes that survived in the mash are halotolerant because of the hypertonic environment in the mash (Kapardar *et al.*, 2010). Brine is crucial in the formation of flavour compounds. Its addition promotes the transition from the koji to the mash stage by affecting the microbial community. *Pediococcus*, *Alistipes*, *Allobaculum*, *Arthrobacter*, *Delftia*, *Marinobacter* and

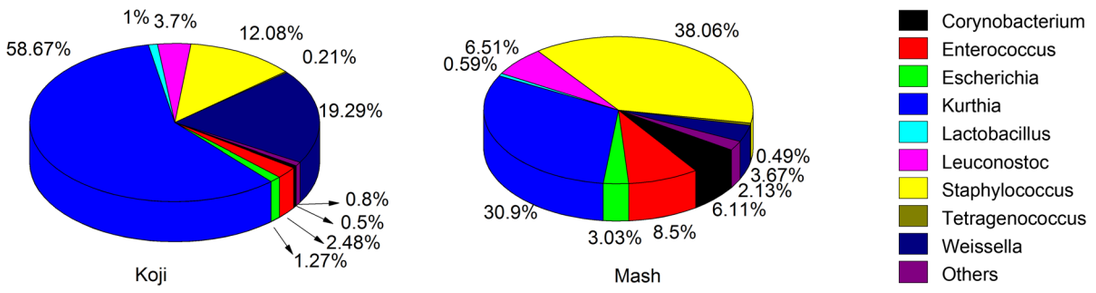

Fig. 3. Genus-level relative abundance and identifications of bacterial community for both koji and mash

Ruminococcus were genera not detected in the mash, suggesting that they are restrained by high salt environment and not necessary for mash fermentation. *Kurthia* was the most abundant bacterial genus in the koji. Its relative abundance decreased when the fermentation entered the mash stage. *Kurthia* sp. produces protease and volatile fatty acids in soy sauce fermentation (Steele *et al.*, 1992; Goodfellow *et al.*, 1980). Given that this genus is strictly aerobic, the insufficient dissolved oxygen in the mash restrains its growth. A previous study revealed that *Staphylococcus* sp. helps produce high levels of volatile fatty acids and makes soy sauce show similar sensory characteristics to long-term fermented fish sauce (Wah *et al.*, 2013). *Staphylococcus* sp. is the major bacteria involved in salted and fermented seafood; it has a strong ability to survive in a hypertonic environment (Guan *et al.*, 2011).

In the present study, the metagenomic method showed that *Weissella*, *Staphylococcus* and *Kurthia* were the dominant genera in the koji. *Micrococcus* spp. and *Bacillus* spp. were previously believed to be the predominant bacteria in the koji (Takazane *et al.*, 1998). Wood indicated that genera *Micrococcus*, *Streptococcus*, *Bacillus* and lactic acid bacteria appear spontaneously in the mash brewed by traditional methods (Wood 1985). By contrast, *Micrococcus* sp. was not detected in the mash in the current study. These inconsistent results may be attributed to the differences in specific production technology, fermentation environment or brewing material.

As far as we know, few studies have used metagenomic methods to investigate microbial community changes during soy sauce fermentation. Tanaka *et al.* have investigated the bacterial community in soy sauce using a PCR-DGGE method and found that *Weissella*, *Salmonella*, *Lactobacillus* and *Staphylococcus* genera were dominant in the koji (Tanaka *et al.*, 2012). Our previous study using PCR-DGGE method (Wei *et al.*, 2013), found that *Staphylococcus* sp. and *Bacillus* sp. were the dominant bacteria and detected in the whole fermentation process of soy sauce, while *Kurthia* sp. and *Klebsiella* sp. appeared in the koji fermentation and fade away in the mash fermentation. By comparison, those results based on DGGE method are similar in the aspect of abundant microbes with these obtained in present study based on metagenomic method. Obviously, metagenomic method reached higher species coverage than DGGE-based method, indicating that metagenomic method could detect the microbes with lower abundance.

This study provided new clues to understand soy sauce fermentation process. It was found that 7 genera disappeared and 12 new genera appeared from koji to mash stage. The dominant bacterial genera in the koji stage were *Kurthia*, *Weissella* and *Staphylococcus*, while those in the mash stage were *Kurthia*, *Enterococcus* and *Leuconostoc*. To the best of our knowledge, this study is the first time to apply metagenomic technology to analyse the bacterial communities involved in soy sauce fermentation. This study will benefit the optimisation of microbial composition and the quality control of fermentation in future research.

Acknowledgements

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SHORT COMMUNICATION

Seroprevalence of Rubella and Cytomegalia in Young Women from Biała Podlaska District

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Abstract

The aim of this study was to analyze the seroprevalence of rubella and cytomegalia among young women. The study included 175 healthy women from the Biała Podlaska District, aged 16 to 35 years. Anti-rubella and anti-CMV IgG were determined by ELISA. 172 (98.3%) study subjects tested positive for rubella antibodies, 1 (0.6%) was seroindeterminate and 2 (1.1%) were seronegative. CMV antibodies were detected in 119 (68.0%) participants; the series included also 1 (0.6%) seroindeterminate and 55 (31.4%) seronegative women. The levels of rubella and CMV antibodies were not significantly affected by age, place of residence and educational level of the study subjects.

Key words: cytomegalovirus, *Rubella virus*, seroprevalence in women

Rubella virus, a pathogen from the *Togaviridae* family, is found solely in humans and causes rubella, a typical childhood disease. The virus is spread *via* airborne droplets containing respiratory secretions from an infected person. Children with rubella present with maculopapular or macular rash and lymph node swelling. Infection in adults is usually more severe, and may be associated with bone and joint pain. The most severe form of infection is congenital rubella associated with primary maternal infection during pregnancy. Congenital rubella may contribute to vision defects, deafness, cardiac anomalies, microcephalia, developmental disorders, fetal death or infant mortality during the 1st year of life. Transfer of maternal antibodies produced in response to infection or immunization protects fetus against rubella (Lambert *et al.*, 2015; Murray *et al.*, 2011).

Human herpesvirus 5 (HHV-5), referred to as human cytomegalovirus (CMV, HCMV), is a lymphotropic virus from the *Herpesviridae* family, replicating in human fibroblasts, epithelial cells and macrophages. CMV establishes a latent infection in T-lymphocytes and macrophages. CMV may be spread with saliva, urine, breast milk, vaginal and cervical secretions, semen and blood. CMV infection may occur in utero; furthermore, the virus may be transmitted *via* oral and sexual route, during transfusions and organ transplantations. In immunocompetent persons, the infection is typically asymptomatic or has the form of a mild mononucleosis-like syndrome. However, it may become more severe in immunocompromised subjects or in patients on immunosuppressive treatment (Ludwig and Hengel, 2009; Murray *et al.*, 2011). Congenital CMV infection is considered to be the most common congenital transmissible disease in Europe. Primary maternal infection during pregnancy poses the highest risk of CMV transmission to the fetus. In seronegative women, the risk of primary infection approximates 1–8%, and in 32% of the cases results in fetal transmission of CMV. Up to 10–18% of infected neonates develop congenital CMV infection which may manifest with impaired psychomotor development, vision disorders, hearing loss or complete deafness. Whereas most of the infected neonates are asymptomatic at birth, 10–15% of them may develop late complications, typically hearing loss. Fetuses of seropositive women are usually protected by maternal CMV antibodies. However, even in such cases there is a 1.4% risk of fetal infection due to reactivation of a latent maternal infection with CMV or superinfection with another viral strain, and up to 8% of

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children infected in utero may suffer from hearing loss (Hamilton *et al.*, 2014; Kadambari *et al.*, 2011; Ludwig and Hengel, 2009).

The aim of the study was to analyze the seroprevalence of *Rubella virus* and cytomegalovirus antibodies among young women. The study included 175 healthy women from the Biała Podlaska District (Lublin Province, Poland), aged between 16 and 35 years (mean age 22.7 years). Women living in towns and villages constituted 46% (n = 80) and 54% (n = 95) of the study group, respectively. Fifty six (32%) study subjects declared having higher education; 118 (67%) women had completed secondary and 1 (1%) primary education. Blood samples were collected in May 2015.

The presence of anti-*Rubella virus* IgG was detected by ELISA (Anti-*Rubella virus* ELISA (IgG), Euroimmun, Germany). Microtiter wells were coated with antigens of *Rubella virus*. The results above or equal 11 relative units/ml (RU/ml) were considered as positive, below 8 RU/ml as negative, whereas borderline results were ≥ 8 and < 11 RU/ml. The presence of anti-CMV IgG was detected by ELISA (Anti-CMV ELISA (IgG), Euroimmun, Germany). Microtiter wells were coated with antigens of CMV. The results above or equal 22 RU/ml were considered as positive, below 16 RU/ml as negative, whereas borderline results were ≥ 16 and < 22 RU/ml. The tests were carried out and the results were interpreted according to the manufacturer's instructions.

The results were subjected to statistical analysis with Statistica v. 10 package. Significant differences in values of quantitative variables were identified with non-parametric Mann-Whitney U-test, with statistical significance threshold set at $p = 0.05$. The study protocol was approved by the Local Bioethics Committee at the Medical University of Lublin (decision no. KE-0254/183/2014).

One hundred seventy two (98.3%) study subjects tested positive for rubella antibodies, 1 (0.6%) was seroindeterminate and 2 (1.1%) were seronegative. CMV antibodies were detected in 119 (68.0%) participants; the series included also 1 (0.6%) seroindeterminate subject and 55 (31.4%) seronegative women. Detailed information about the antibody levels is presented in Table I. The levels of rubella and CMV antibodies were not significantly affected by age, place of residence and educational level of the study subjects.

According to the ECDC report on epidemiology of mumps and rubella, a total of 1 708 rubella cases were recorded in Europe between July 2015 and June 2016, and the vast majority of them (n = 1 553, 91%) were diagnosed in Poland. However, as emphasized by the authors of the report, only a very small proportion of the diagnoses (1.1%) were confirmed in a laboratory setting (ECDC 2016). In 1989–2002, active immunization with a monovalent rubella vaccine was obligatory solely for Polish girls. However, beginning in 2003, all Polish children, both girls and boys, need to be immunized with two doses of MMR (measles, mumps, rubella) combined vaccine at 2 and 10 years of age (Zimmerman *et al.*, 2011). According to the report published by the National Institute of Hygiene, MMR vaccination coverage in Poland is high, exceeding 95% in 2015 (Czarkowski *et al.*, 2015). Smits *et al.* (2014) presented a 32-year Dutch experience with MMR vaccine; the product proved to be effective, as long-term persistence of antibodies induced by vaccination was demonstrated in up to 95% of subjects who had received two doses of MMR.

The study conducted in 2000–2002 by Wysokińska *et al.* (2004) in a group of 15- to 30-year-old women (n = 1 289) from various regions of Poland, documented the presence of anti-rubella IgG > 15 IU/ml in 89.5% of the subjects. Also 98.3% of women participating in our study had rubella antibody levels ≥ 11 RU/ml. Currently, ≥ 10 IU/ml is considered a protective level of rubella antibodies (Lambert *et al.*, 2015). In view of the abovementioned findings, a considerable proportion of Polish children diagnosed with rubella solely on the basis of clinical presentation, may in fact suffer from other viral diseases that manifest with maculopapular rash, *e.g.* parvovirus B19 infection.

Table I
Titers of anti-*Rubella virus* and anti-CMV IgG depending on age

Age	Number of persons (N)	Titers of anti-*Rubella virus* (RU/ml)				Titers of anti-CMV (RU/ml)			
		$\bar{x}$	SD	MIN	MAX	$\bar{x}$	SD	MIN	MAX
≤ 19	17	55.78	24.42	10.40	97.09	59.53	60.89	< 2.00	167.78
20–21	73	82.48	53.21	6.96	> 200.00	77.02	62.92	< 2.00	> 200.00
22–23	40	76.34	48.18	5.09	> 200.00	77.58	64.63	< 2.00	> 200.00
24–25	14	117.14	63.63	29.00	> 200.00	85.94	69.81	< 2.00	> 200.00
26–27	10	87.18	53.04	20.93	191.93	63.89	71.81	< 2.00	199.14
≥ 28	21	98.02	60.46	11.21	> 200.00	104.13	75.23	< 2.00	> 200.00
Total	175	83.39	53.10	5.09	> 200.00	78.67	65.79	< 2.00	> 200.00

The seroprevalence of CMV antibodies among women of childbearing age ranges between 45% and 100%. The highest seroprevalence is reported in Africa, Asia and South America, and the lowest in Western Europe and United States. The seroprevalence increases with age; furthermore, it was shown to be higher in individuals with poor socioeconomic status and in non-white women (Cannon *et al.*, 2010). The seroprevalence of CMV antibodies in European women of childbearing age varies between 30% and 96%. The lowest seroprevalence rates were documented in Ireland (in native Irish women), Netherlands and Germany, and the highest in Turkey, United Kingdom in Asian women and Ireland in non-Irish immigrants (Ludwig and Hengel, 2009).

Gaj *et al.* (2012) analyzed the seroprevalence of CMV antibodies in pregnant women who have been hospitalized in two clinics in Łódź in 1999–2009. Anti-CMV IgG and IgM were detected in 76.7% and 13% of the study subjects, respectively. The seroprevalence of CMV antibodies was not influenced by age, parity and place of residence; however, anti-CMV IgG were detected less often in women examined in 2005–2009 than in those tested during the earlier period. In another study, conducted in 2010–2011, also among pregnant women from two hospitals in Łódź, Wujcicka *et al.* (2014) documented a 62.4% seroprevalence of anti-CMV IgG. The CMV antibodies were significantly more often found in women aged 36 years and older, with primary or vocational education, and in those having children. Siennicka *et al.* (2016) analyzed sera from 712 women aged between 15 and 49 years, and showed that the seroprevalence of CMV antibodies increased with age, from 74.3% in subjects younger than 30 years, to 94.3% in those older than 45 years. The seroprevalence was not affected by the place of residence of the study subjects. In our present study, the seroprevalence of anti-CMV IgG amounted to 68.0%. We did not observe statistically significant age-related differences in the antibody levels, probably due the fact that all the study subjects were younger than 36 years and most of them (92%) had no children. Nevertheless, these findings imply that Poland is a country with a moderate seroprevalence of CMV antibodies.

In conclusion, this study documented the high, up to 98.3% seroprevalence of rubella antibodies, which seems to confirm the effectiveness of the vaccination program that is currently used in Poland. 68.0% of the study subjects tested positively for anti-CMV IgG, which implies that Poland is a country with a moderate seroprevalence of cytomegalovirus antibodies.

Acknowledgements

We express our gratitude to Marta Fiedoruk and Karol Laskowski for their assistance in collection of the study material. The study was supported from funds of Pope John Paul II State School of Higher Education in Biała Podlaska.

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Dear Authors,

Presidium of the Main Board of the Polish Society of Microbiologists voted on 9.03.2017 to add 23% VAT to the publication fee. Therefore, the fee is 250 USD + 23% VAT for non-members of Polish Society of Microbiologists and 125 USD + 23% VAT for corresponding authors, who are PTM members.

New payment rules apply for manuscripts printed in issue 1/2018 and onwards, including all manuscripts accepted in 2017, scheduled to be published in 2018.

Polish Society of Microbiologists

ERRATA

Pol J Microbiol 2017; 66(2): 151–161 Biochar-rhizosphere interactions – a review
SŁAWOMIR GŁUSZEK, LIDIA SAS-PASZT, BEATA SUMOROK and RYSZARD KOZERA

On page 151 there is an error in affiliation.

The affiliation of Ryszard Kozera is
Warsaw University of Life Sciences – SGGW, Warsaw, Poland

Konferencja pod patronatem PTM

IV edycja konferencji
„Wektory i patogeny – w przeszłości i przyszłości"
Wrocław, 24 listopada 2017

Szanowni Państwo,

Instytut Genetyki i Mikrobiologii Uniwersytetu Wrocławskiego oraz Wrocławski Oddział Polskiego Towarzystwa Mikrobiologów i Wrocławski Oddział Polskiego Towarzystwa Parazytologicznego zapraszają na IV edycję konferencji pt. „*Wektory i patogeny – w przeszłości i przyszłości*".

Konferencja ma na celu prezentację badań z zakresu mikrobiologii i parazytologii jakie są prowadzone aktualnie w krajowych jak i zagranicznych jednostkach naukowych. Pragniemy również udokumentować historyczny dorobek polskich naukowców w tych dziedzinach. W tym roku szczególną uwagą objęty będzie problem uwarunkowanych środowiskowo chorób infekcyjnych i inwazyjnych, których czynnikami etiologicznymi są patogeny transmitowane przez stawonogi (wektory), głównie hematofagiczne kleszcze i komary, a także ukazanie skutecznych sposobów zapobiegania i monitorowania tych zagrożeń.

Podczas konferencji planowana jest prezentacja praktycznych osiągnięć 20-letniej współpracy Instytutu Genetyki i Mikrobiologii UWr z Wydziałem Środowiska i Rolnictwa Urzędu Miasta Wrocławia w zakresie biologicznego (mikrobiologicznego) zwalczania komarów na terenie Aglomeracji Wrocławskiej. Ważnym celem konferencji jest także integracja środowiska naukowego oraz ukazanie osiągnięć naukowych młodych adeptów nauki.

Szczegółowe informacje zamieszczone są na stronie:
http://www.mikrobiologia.uni.wroc.pl

Organizator:
Uniwersytet Wrocławski, Instytut Genetyki i Mikrobiologii

Miejsce:
Uniwersytet Wrocławski, Instytut Genetyki i Mikrobiologii
ul. Przybyszewskiego 63/77, 51-148 Wrocław

Konferencja pod patronatem PTM

V edycja konferencji
Viruses of Microbes V: Biodiversity and future applications
Wrocław, 9–13 lipca 2018

Jest to piąte już spotkanie z serii międzynarodowych konferencji towarzystwa International Society for Virus of Microbes (ISVM) poświęconej wirusom drobnoustrojów.

Konferencje Virus of Microbes dobywają się co dwa lata, a rozpoczęły się w 2010 roku w Instytucie Ludwika Pasteura w Paryżu, a następnie kontynuowane były w Brukseli (2012), Zurichu (2014), i Liverpoolu (2016), gromadząc na każdym spotkaniu ok. 400–500 uczestników z całego świata.

Przyszłoroczne spotkanie zatytułowane jest „"Biodiversity and future application". Tematyka pięciodniowej konferencji poświęcona będzie podstawowym i aplikacyjnym badaniom naukowym nad wirusami mikroorganizmów (algi, archaea, bakterie, grzyby, pierwotniaki i wirusy). Wirusy są kluczowym elementem warunkującym bioróżnorodność i ewolucję mikrobiologiczną, jak również służą jako narzędzie w biologii molekularnej. W ostatnich czasie coraz większym zainteresowaniem cieszą się badania nad bakteriofagami, które stanowią obiecująca alternatywę leczenia infekcji wywołanych zwłaszcza przez wielolekooporne szczepy patogenów człowieka, a są stosowane już w konserwacji żywności, hodowli zwierząt i produkcji roślin uprawnych.

Uniwersytet Wrocławski (prof. dr hab. Zuzanna Drulis-Kawa) wraz z Laboratorium Bakteriofagowym Instytutu Immunologii i Terapii Doświadczalnej we Wrocławiu (prof. dr hab. Krystyna Dąbrowska), mają zaszczyt organizować kolejną międzynarodową edycję tej konferencji w 2018 roku.

Konferencja porusza tematykę niezwykle aktualną i będzie dedykowana do wszystkich osób zajmujących się zagadnieniami z zakresu zwalczania zakażeń bakteryjnych, jak również ekologii i różnorodności biologicznej drobnoustrojów.

Konferencja została objęta patronatem przez European Molecular Biology Organization jako EMBO Workshop „Viruses of microbes 2018" http://www.embo.org/events

Lokalizacja:
Uniwersytet Wrocławski, Wydział Prawa, Administracji i Ekonomii, ul. Uniwersytecka 22/26, 50-145 Wrocław

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VIRUSES OF MICROBES V
Biodiversity and future applications

9–13 July 2018 Wrocław, Poland

Institute of Genetics and Microbiology
University of Wrocław
&
Hirszfeld Institute of Immunology and Experimental Therapy
Polish Academy of Sciences

On behalf of the organising committee of the ISVM conference on the Viruses of Microbes, we are pleased to invite to the fifth meeting in an international series that began in 2010 at the Pasteur Institute.

This event is one that focuses on basic and applied scientific research on viruses infecting microbes (algae, archaea, bacteria, fungi, protozoa and viruses). Viruses have always been a key element of microbial diversity and evolution, as well as a tool for the molecular biologist to learn more about how the host cell functions, but this information has also been put to productive use in latter days to control infections and fouling in many areas of our modern life.

The conference is included to the EMBO Workshop list http://www.embo.org/events

Location:
Uniwersytet Wrocławski, Wydział Prawa, Administracji i Ekonomii
ul. Uniwersytecka 22/26, 50-145 Wrocław

Organizers:
Uniwersytet Wrocławski; Instytut Immunologii i Terapii Doświadczalnej PAN

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INFORMACJA O PRACY PREZYDIUM ZARZĄDU GŁÓWNEGO PTM

1) Większość członków Prezydium była za przyjęciem **Uchwały nr 26-2017 z dnia 30.08.2017 r. w sprawie stanowiska PTM odnośnie przyznania absolwentom uniwersyteckich studiów magisterskich na kierunku Mikrobiologia prawa wykonywania zawodu Diagnosty Laboratoryjnego.** Poniżej zamieszczony jest tekst, który będzie opublikowany w czasopismach PTM. Informujemy, że nasze stanowisko zostało przekazane listownie do kancelarii Pana Prezydenta RP Andrzeja Dudy, Pani Premier Beaty Szydło, Panów Ministrów ds. Zdrowia Konstantego Radziwiłła i ds. Nauki Jarosława Gowina. Od Pana Ministra K. Radziwiłła otrzymaliśmy odpowiedź, że prace nad ustawą są prowadzone i nasze stanowisko będzie wzięte pod uwagę.

Stanowisko Polskiego Towarzystwa Mikrobiologów
w sprawie przyznania absolwentom uniwersyteckich studiów magisterskich na kierunku Mikrobiologia
prawa wykonywania zawodu Diagnosty Laboratoryjnego

W oparciu o Rozporządzenie Ministra Zdrowia z dnia 14 listopada 2008 r. (Dz. U. Nr 208, Poz. 1312), wpisujące mikrobiologię na listę dziedzin mających zastosowanie w ochronie zdrowia oraz ustawę Prawo o szkolnictwie wyższym z 2011 r. (Dz. U. Nr 84, poz. 455), utworzono niezależne kierunki Mikrobiologia na wydziałach pięciu Polskich uniwersytetów posiadających odpowiednie uprawnienia (Uniwersytetu Łódzkiego, Szczecińskiego, Warmińsko-Mazurskiego, Wrocławskiego oraz Zachodniopomorskiego Uniwersytetu Technologicznego). Opracowano standard kształcenia dla kierunku Mikrobiologia, dokonując przesunięcia profilu kształcenia z ogólnobiologicznego w kierunku ogólnomedycznym, co wydaje się, że powinno zapewnić zdobywanie przez studentów kierunku Mikrobiologia wiedzy i umiejętności upoważniających do podejmowania samodzielnych czynności w ramach szeroko rozumianej medycznej diagnostyki laboratoryjnej. Standard ten został opracowany przez mikrobiologów, immunologów i genetyków z uniwersytetów: Łódzkiego, Warszawskiego, Wrocławskiego i UMCS w Lublinie, przy współpracy z członkami Komitetu Mikrobiologii PAN oraz w porozumieniu z Krajową Radą Diagnostów Laboratoryjnych oraz kierownikami laboratoriów medycznych, i zatwierdzony przez Radę Główną Nauki i Szkolnictwa Wyższego. Na podstawie ww. znowelizowanej ustawy Prawo o Szkolnictwie Wyższym, standard ten został przepisany na odpowiednie efekty kształcenia w zakresie wiedzy, umiejętności i kompetencji społecznych.

Podczas pięcioletnich, dwustopniowych studiów (3+2), osoby uzyskujące najpierw licencjat na kierunku Mikrobiologia, a następnie tytuł zawodowy magistra Mikrobiologii powinni uzyskać efekty kształcenia wymagane do wykonywania zawodu Diagnosty Laboratoryjnego określone w Rozporządzeniu Ministra Nauki i Szkolnictwa Wyższego z dnia 24 sierpnia 2016 r. (Poz. 1434), które mają obowiązywać od roku akademickiego 2017/2018 studentów kierunku Analityka Medyczna/Medycyna Laboratoryjna. Studenci obu stopni studiów kierunku Mikrobiologia uzyskują wszystkie wymagane tym Rozporządzeniem i weryfikowane podczas egzaminów i kolokwiów zaliczeniowych, efekty kształcenia. Biorąc pod uwagę odpowiedzialność, jaka spoczywa na zawodzie Diagnosty Laboratoryjnego, wyrażamy przekonanie, iż tylko osiąganie określonych standardów/efektów kształcenia w ramach wybranych kierunków studiów wyższych, może być gwarancją przygotowania absolwentów do pracy w tym zawodzie, zapewniając jednocześnie bezpieczeństwo pacjentów i prawidłową współpracę diagnostów z lekarzami.

W związku z powyższym większość członków Polskiego Towarzystwa Mikrobiologów uważa, iż uprawnienia do tytułu Diagnosty Laboratoryjnego powinny być uzależnione od odpowiednich efektów kształcenia, które muszą być ustalone dla każdego kierunku, a nie od nazwy kierunku studiów. Absolwenci uniwersyteckich kierunków Mikrobiologia, z tytułem magistra mikrobiologii, powinni mieć prawo do starania się o wpis na Listę Diagnostów Laboratoryjnych, ponieważ podczas studiów uzyskują efekty kształcenia wymagane do wykonywania zawodu Diagnosty Laboratoryjnego, zgodne ze standardem przewidywanym dla kierunku Analityka Medyczna/Medycyna Laboratoryjna, dokumentowane dyplomem, dorobkiem (praca licencjacka, magisterska, doktorska, publikacje) oraz odbytymi praktykami i stażami zawodowymi. Prawo wpisu na Listę Diagnostów Laboratoryjnych dla absolwentów kierunku Mikrobiologia można by uzyskać po ukończeniu studiów i po rocznym stażu w wieloprofilowym laboratorium medycznym oraz zdaniu egzaminu państwowego.

2) Większością głosów podjęto **Uchwałę nr 39-2017 z dnia 31.10.2017 r., w której wyrażono pozytywną decyzję o objęciu patronatem przez PTM konferencji „Virus of Microbes, 2018" zaplanowanej w dniach 9–13 lipca 2018 r. we Wrocławiu, pod warunkiem uzyskania zniżki 10% (co najmniej 100 zł) na opłatę konferencyjną dla członków PTM.**

3) Wydawanie Polish Journal of Microbiology jest bardzo kosztowne, jakość naukowa szeregu nadsyłanych manuskryptów jest niska. W uzgodnieniu z Redaktor Naczelną PJM niezbędne jest wprowadzenie szeregu istotnych zmian w funkcjonowaniu czasopisma. Wychodząc z założenia, że PJM powinno się samofinansować, a ponadto wsłuchując się w głosy delegatów PTM obecnych na Konferencji 90 lat PTM postulujących, aby część środków finansowych PTM udostępnić Oddziałom Terenowym, postanowiono w **Uchwale nr 40-2017 z dnia 31.10.2017 r., przyjętej większością głosów członków Prezydium ZG PTM, aby dla manuskryptów otrzymywanych przez redakcję PJM od 01.07.2018 r. podnieść opłaty redakcyjne za publikację w PJM, odpowiednio: dla autorów korespondencyjnych będących członkami PTM z: 125 USD + 23% VAT do 250 USD + 23% VAT oraz dla pozostałych osób z 250 USD + 23% VAT do 500 USD + 23% VAT.**

4) Ponadto uważamy, że Polish Journal of Microbiology posiada niewykorzystany potencjał naukowy. Proces redakcyjny i wydawniczy prowadzony na wysokim poziomie za pomocą specjalistycznego oprogramowania edytorskiego, połączony z odpowiednią reklamą czasopisma, powinien przynieść wzrost cytowania artykułów PJM i zwiększenie współczynnika Impact Factor. Redakcja PJM korzysta obecnie z firmy edytorskiej Index Copernicus, jednakże współpraca z tą firmą w coraz większym zakresie nie spełnia podstawowych potrzeb i oczekiwań czasopisma, co skutkuje opóźnieniami w procesie wydawniczym i opóźnieniemi w publikacji wersji internetowej artykułów. Ponadto Firma edytorska Index Copernicus, z którą źle się współpracuje redakcji PJM, zmienia aktualnie system edytorski, co powoduje bałagan związany z funkcjonowaniem obu systemów edytorskich i widać, że nowy system ma szereg wad. W wyniku rozmów prowadzonych przy współudziale redakcji PJM, z dwiema firmami wydawniczymi, których koszt byłby do zaakceptowania przez PTM, uznano, że firma EXELEY INC. z Nowego Jorku, Stany Zjednoczone, byłaby najbardziej odpowiednia i rokująca nadzieję na poprawę funkcjonowania PJM. **W związku z powyższym jednomyślnie podjęto Uchwałę nr 41-2017 z dnia 01.11.2017 r. w sprawie zmiany firmy edytorskiej umożliwiającej pracę redakcji Polish Journal of Microbiology w profesjonalnym systemie edytorskim oraz wydającej wersję elektroniczną PJM i podpisania umowy na okres 3 lat na wydawanie PJM on-line. Rozmowy z firmą EXELEY INC są zaawansowane i zmierzają do podpisania umowy na nowy system wydawniczy, który byłby uruchomiony od 01.01.2018 r.**

5) Dwaj członkowie PTM otrzymali Granty FEMS na wyjazdy do naukowych ośrodków zagranicznych. Złożone zostały 2 wnioski o granty FEMS dofinansowujące wyjazdy na konferencje naukowe.

6) Podjęliśmy współpracę z Kancelarią Prawną w celu uzyskania opinii odnośnie możliwości powołania Pełnomocników Delegatów na Nadzwyczajne Walne Zgromadzenie Delegatów PTM podczas Konferencji 90 lat PTM. Umożliwiło to uzyskanie quorum na Walnym Zgromadzeniu Delegatów wymaganego do wprowadzania zmian w Statucie PTM. Po uzyskaniu wszystkich materiałów z NWZD, z pomocą Kancelarii Prawnej zostanie przygotowany wniosku do sądu (KRS), który zatwierdzi proponowane zmiany Statutu PTM.

7) Bardzo dużo pracy i wysiłku poświęciliśmy na przygotowanie i organizację naszej Konferencji „PTM wczoraj – dziś – jutro". Odbyła się ona w dniach 22–23 września 2017 roku w Auditorium Maximum Uniwersytetu Jagiellońskiego. Współorganizatorem Konferencji był Uniwersytet Jagielloński; Wydział Biochemii, Biofizyki i Biotechnologii oraz Katedra Mikrobiologii Wydziału Lekarskiego. Konferencja została zorganizowana z okazji przypadającej w tym roku 90-tej rocznicy powołania Polskiego Towarzystwa Mikrobiologów, 160-lecia urodzin Profesora Odona Bujwida – Ojca mikrobiologii polskiej i twórcy Katedry Higieny na Uniwersytecie Jagiellońskim, a także z okazji 130 rocznicy wygłoszenia przez Profesora na Uniwersytecie Jagiellońskim słynnych „Pięciu odczytów o Bakteryach" uznanych za pierwszy wykład z mikrobiologii i opublikowanych jako pierwszy polski podręcznik mikrobiologii. W konferencji wzięło udział 217 osób, w tym 54 osoby do 35 roku życia, które otrzymały wsparcie finansowe FEMS. Tematyka naukowa Konferencji, wraz z częścią poświęconą historii polskiej mikrobiologii oraz upamiętnieniu działalności i zasług Prof. Odona Bujwida, dała możliwość zaprezentowania współczesnych osiągnięć z różnych dyscyplin mikrobiologii. Odbyły się sesje wykładowe z zakresu mikrobiologii lekarskiej, weterynaryjnej, przemysłowej, żywności i środowiska. Wyniki badań naukowych były również prezentowane w trakcie sesji plakatowych, na których przedstawiono 148 plakatów. Uczestnicy konferencji otrzymali reprint wygłoszonych wykładów Prof. O. Bujwida, zamieszczonych w publikacji „PIĘĆ ODCZYTÓW O BAKTERYACH Rys ogólnych zasad bakteryologii w zastosowaniu do chorób zakaźnych z dołączeniem uwag o surowicach leczniczych, szczepieniach ochronnych i dezynfekcyi z tablicą zdjęć mikro-fotograficznych własnych" wydanie trzecie znacznie poszerzone, opublikowane w 1907 roku, Skład Główny w księgarni Gebethnera i Sp.

Konferencja była także okazją do zaprezentowania działalności i historii 14 terenowych oddziałów PTM.

SEKRETARZ
Polskiego Towarzystwa Mikrobiologów
dr n. farm. Agnieszka E. Laudy

PREZES
Polskiego Towarzystwa Mikrobiologów
prof. dr hab. Stefan Tyski

CZŁONKOWIE WSPIERAJĄCY PTM

Członek Wspierający PTM – Złoty
od 27.03.2017 r.

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od 07.06.2017 r.

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Aesculap Chifa Sp. z o.o. jest członkiem grupy B. Braun, jednej z wiodących na świecie firm medycznych, produkującej i dystrybuującej miedzy innymi preparaty do antyseptyki rąk, skóry, błon śluzowych, do mycia i dezynfekcji wyrobów medycznych oraz powierzchni.

Członek Wspierający PTM – Srebrny
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Członek Wspierający PTM – Srebrny

Od ponad 100 lat siedziba Wodociągów Krakowskich mieści się przy ul. Senatorskiej. Budowę obiektu ukończono w 1913 roku. W 2016 r. do sieci wodociągowej wtłoczono ponad 56 mln m^3 wody.
Szacuje się, że ponad 99,5% mieszkańców Gminy Miejskiej Kraków posiada możliwość korzystania z istniejącej sieci wodociągowej.

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